University of Alberta

Vitality and Mortality of *Candida albicans*

by

Robert Steven Liao ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Medical Microbiology and Immunology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Vitality and Mortality of *Candida albicans* submitted by Robert Steven Liao in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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August 16, 2002
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Quotes

1 In the middle of our life's way
I found myself in a wood so dark
That I couldn't tell where the straight path lay.
4 Oh how hard a thing it is to embark
Upon the story of that savage wood,
For the memory shudders me with fear so stark
7 That death itself is hardly a more bitter food;
Yet whatever I observed there I'll convey,
In order to tell what I found that was good.

Inferno, canto 3, 1.1

Inigo: He's dead. He can't talk.
Max: Oooooohhh! Look who knows so much, eh! It just so happens that your friend here is only mostly dead. There's a big difference between mostly dead and all dead. Please open his mouth. [*He inserts the bellows*] Now, mostly dead is slightly alive. Now, all dead... well, with all dead, there's usually only one thing that you can do.
Inigo: What's that?
Max: Go through his clothes and look for loose change. [*Max pumps air into Westley and yells at him*] Hey! Hello in there! Hey! What's so important? Whatcha got here, that's worth living for? [*Max pushes on Westley's chest*] **Westley:** [*barely audible*] True...love....

From the script of the Princess Bride

MORTICIAN:Bring out your dead! Bring out your dead!
[clang] Bring out your dead!
[clang] Bring out your dead!
[clang] Bring out your dead!
CUSTOMER: Here's one -- nine pence.
DEAD PERSON: I'm not dead!
MORTICIAN: What?
CUSTOMER: Nothing -- here's your nine pence.
DEAD PERSON: I'm not dead!
MORTICIAN: Here – He says he's not dead!
CUSTOMER: I'm not.
DEAD PERSON: He isn't.
MORTICIAN: Oh, I can't take him like that -- it's against regulations.

From Scene 2 of Monty Python's The Holy Grail

ABSTRACT

Invasive infection with *Candida albicans* is a major source of morbidity and mortality. Amphotericin B and fluconazole are the antifungal agents most commonly used to treat serious *C. albicans* infections. Therapy with amphotericin is often prolonged and refractory, and the correlation between MICs and clinical outcome is uncertain. Endpoint determination for fluconazole susceptibility testing is also problematic and a major source of error.

We hypothesized that by studying *C. albicans* viability we could improve our understanding of why amphotericin B is less effective than would be expected of a fungicidal antifungal agent, and also correlate these insights to improving antifungal susceptibility testing. A number of assays were developed that allowed for a unique overall view of *C. albicans* viability by separately assessing parameters directly associated with vitality and mortality. The loss of *C. albicans* viability was shown to be a gradual process that could be delineated into different stages between alive and dead. The loss of replication and the ability to form colony forming units was shown to be an early event in the death of *C. albicans*. *In vitro* incubation with amphotericin B, at concentrations usually achieved in the serum with normal dosing, showed that *C.*

albicans exists in a viable nonreplicating state that was capable of resuscitation and growth after additional incubation for 15 hr at 22°C. This capacity to recover and grow *in vitro* indicated that amphotericin B should not be considered fungicidal at 0.5 μg/mL. Sequential combination treatment with amphotericin *in vitro* followed by miconazole was shown to be effective in achieving fungicidal inhibition and eliminating this rescue mechanism.

Several vitality assays were also integrated into the current methods used to assess antifungal susceptibility testing in order to improve endpoint determinations. Measurement of *C. albicans* intracellular adenosine 5'-triphosphate allowed for rapid susceptibility testing with amphotericin B within 30 minutes. The fluorescent dye, 5,6-carboxyfluorescein diacetate, was also incorporated into the microdilution method and was shown to provide objective and quantifiable endpoints for susceptibility testing a wide range of antifungal agents at 24 h and 48 h that were reproducible and easier to interpret than the existing methods.

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TABLE OF CONTENTS

Chapter 1: Introduction **Page**

PART ONE: *Candida albicans* and Antifungal Therapy

I. Introduction to *Candida albicans*.....2

II. Opportunistic Human Pathogen.....4

III. Epidemiology of 'An Emerging Threat'..... 7

IV. Difficulties with Diagnosing Candidemia.....8

V. Systemic Antifungal Therapy..... 10

VI. *In vitro* Antifungal Susceptibility Testing..... 19

PART TWO: Viability, Killing and Injury

VII. Replication Competency.....26

VIII. Cellular Integrity and Metabolic Potential..... 27

IX. Loss of Replication and Recovery from Sublethal Stress.....29

X. *C. albicans* Replication Competency and Viability: Observations in Clinical Microbiology..... 31

Page

XI. Rationale and Hypothesis..... 33

XII. Table..... 36

XIII. Figure..... 37

XIV. References..... 38

Chapter 2: A Novel Fluorescent Broth Microdilution Method for Fluconazole Susceptibility Testing of *Candida* species

I. Introduction..... 58

II. Materials and Methods..... 59

III. Results..... 63

IV. Discussion..... 65

V. Tables..... 70

VI. Figures..... 72

VII. References..... 76

Chapter 3: Comparative Evaluation of a New Fluorescent CFDA-modified Microdilution Method for Antifungal Susceptibility testing of *Candida albicans* **Page**

I. Introduction..... 81

II. Materials and Methods..... 82

III. Results..... 87

IV. Discussion..... 91

V. Tables..... 97

VI. Figures..... 101

VII. References..... 105

Chapter 4: The Measurement of Intracellular ATP for the Rapid Susceptibility Testing of *Candida albicans* to Amphotericin B and Fluconazole

I. Introduction..... 110

II. Materials and Methods..... 111

III. Results..... 117

IV. Discussion..... 121

Page

V. Figures.....126

VI. References..... 132

Chapter 5: Assessment of the Effect of Amphotericin B on the Vitality and Mortality of *Candida albicans*

I. Introduction..... 137

II. Materials and Methods..... 139

III. Results..... 144

IV. Discussion..... 148

V. Table..... 158

VI. Figures.....160

VII. References..... 170

Chapter 6: Sublethal Injury with Resuscitation or Killing of *Candida albicans* after Amphotericin B Treatment

I. Introduction..... 174

Page

II. Materials and Methods..... 172

III. Results and Discussion..... 178

IV. Table..... 190

V. Figures..... 192

VI. References..... 204

Chapter 7: Discussion

I. Summary..... 209

II. Sublethal Injury and Resuscitation..... 213

III. Sequential Combination Therapy..... 216

IV. Amphotericin B Postantifungal Effect..... 221

V. Amphotericin B: Fungicidal versus Fungistatic..... 225

VI. Amphotericin B Mechanism of Action..... 228

VII. Future Directions..... 232

VIII. Conclusions..... 236

IX. Figure..... 238

X. References..... 240

Appendix I: Materials and Methods **Page**

I. Antifungal Drugs.....249

II. Fungal Culture.....249

III. References..... 252

LIST OF TABLES

Chapter 1 **Page**

Table 1.1 Conditions and therapies that predispose individuals to invasive *Candida* infections..... 36

Chapter 2

Table 2.1 Distribution of difference between the MICs of fluconazole for 41 strains of *C. albicans* determined by comparison of the MIC_{cfda} method to the MIC_{visual} reference method..... 71

Chapter 3

Table 3.1 Comparison between microdilution MICs for *C. albicans* after 24 hr and 48 hr determined by MIC_{visual}, MIC_{spec}, MIC_{alamar}, and MIC_{cfda} methods..... 98

Table 3.2 Summary of the distribution of differences between microdilution MIC methods..... 99

Table 3.3 Distribution of difference between the MIC methods with the highest agreement and showing individual antifungal agents..... 100

Chapter 5

Table 5.1 Summary of four physiological states of *C. albicans* after incubation with increasing concentrations of AMB for 10 hr..... 159

Chapter 6

Table 6.1 Summary of plate counts and mortality-specific staining for *C. albicans* —MY2417 incubated with AMB for 10 hr followed sequentially with a second antifungal agent..... 191

LIST OF FIGURES

Chapter 1 **Page**

Figure 1.1 Structures of systemic antifungal agents..... 37

Chapter 2

Figure 2.1 Range of fluconazole MICs for 52 isolates of *C. albicans* at 24 h and 48 h..... 73

Figure 2.2 Comparison of the effect of fluconazole on representative strains of *C. albicans* using the $MIC_{M27\text{-}A}$ and MIC_{CFDA} methods at 24 hr and 48 hr..... 74

Chapter 3

Figure 3.1 Fluconazole MIC endpoints at 24 hr and 48 hr for a low-high phenotype strain using the MIC_{visual}, MIC_{spec}, MIC_{alamar}, and MIC_{cfda} methods.....102

Figure 3.2 The MIC_{cfda} method for 3 representative strains of *C. albicans* using the antifungal agents: 5FC,

Page

AMB, KTC, VRC, FLC and ITC..... 104

Chapter 4

Figure 4.1 Standard curve of replication competency versus luminescence..... 127

Figure 4.2 Standard curve of luminescence versus ATP concentration..... 128

Figure 4.3 MIC_{atp} method for susceptibility testing of three strains of *C. albicans* to fluconazole at 24 hr and 48 hr..... 129

Figure 4.4 CFDA staining, intracellular ATP, and CFUs for a low-high phenotype strain treated with fluconazole for 12, 24, and 48 hr..... 130

Figure 4.5 MIC_{atp} method susceptibility testing of *C. albicans* to amphotericin B at 30 min, 6 hr and 48 hr..... 131

Chapter 5 **Page**

Figure 5.1 Evaluation of *C. albicans* MY894 for CFU/mL intracellular ATP, and CFUs after 10 hr incubation with amphotericin B..... 161

Figure 5.2 Evaluation of *C. albicans* 96-90 CFU/mL, particles, and ATP with increasing concentrations of amphotericin B..... 162

Figure 5.3 Vitality-specific and mortality-specific fluorescence with amphotericin B at 10 hr..... 163

Figure 5.4 Time-kill curves with amphotericin B showing mortality-specific fluorescence at 1.5, 4.5 and 10 hr..... 164

Figure 5.5 Time-kill curve with amphotericin B at 10 hr showing mortality-specific fluorescence and CFU/mL..... 165

Page

Figure 5.6 Scanning electron micrograph of untreated *C. albicans* growth control at 10 hr at 35°C..... 166

Figure 5.7 Scanning electron micrograph after treating *C. albicans* with 1.2 μg/mL of amphotericin B for 10 hr at 35°C..... 168

Chapter 6

Figure 6.1 Fluorescent staining, ATP, XTT, CFU/mL and particle counts of *C. albicans* MY2417 incubated with amphotericin B for 10 hr at 35°C followed by 15 hr at 22°C..... 193

Figure 6.2 Fluorescent staining, ATP, XTT, CFU/mL and particle counts of *C. albicans* MY2417 incubated with amphotericin B for 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL miconazole..... 197

Page

Figure 6.3 SYBR Green I staining after incubation with amphotericin B for 10 hr followed by: (i) 15 hr at 22°C, (ii) 15 hr at 22°C with the same AMB concentration as at 10 hr, (iii) 15 hr at 22°C with 20 μg/mL of miconazole..... 201

Figure 6.4 Summary time-kill curves with 10 hr exposure to amphotericin B and the following sequential treatment with 20 μg/mL of miconazole for 15 hr at 22°C, showing CFDA, XTT, SYBR Green I, and $DiBAC_4(3)$..... 202

Chapter 7

Figure 7.1 A concept chart for *C. albicans* viability..... 239

ABBREVIATIONS

AMB	amphotericin B
ATP	adenosine 5'-triphosphate
ATTC	American Type Culture Collection
CFDA	5,6-carboxyfluorescein diacetate
CFU	colony forming units
$DiOC_6(3)$	3,3-dihexyloxacarbocyanine iodide
$DiBAC_4(3)$	bis-(1,3-dibutylbarbituric acid)trimethine
DMSO	dimethyl sulfoxide
5FC	5-flucytosine
FLC	fluconazole
ITC	itraconazole
KTC	ketoconazole
MIC	minimum inhibitory concentration
MFC	minimum fungicidal concentration
MOPS	3-[morpholino]propanosulfonic acid-sodium
MTT	1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan
NCCLS	National Committee for Clinical Laboratory Standards
RFU	relative fluorescent units
RLU	relative luminescent units

SDA	Sabouraud dextrose agar
TTC	2,3,5-triphenyl tetrazolium chloride
VRC	voriconazole
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide

Chapter 1

INTRODUCTION

PART ONE: *Candida albicans* and Antifungal Therapy

I. Introduction to *Candida albicans*

I A. Dimorphic Fungus

Candida albicans is the most common fungal pathogen isolated from human patients (198, 52). *C. albicans* is dimorphic and has the ability to replicate by budding as blastoconidia or as hyphae (135). Blastoconidia are oval-shaped and unicellular with a diameter of 2 to 3 μM and a length of 4 to 6 μM (135). Pseudohyphae are formed when replication occurs sequentially in a linear fashion without separation (74). True hyphae differ from pseudohyphae in having septa that separate each cell of the hyphal chain (74). Several factors have been associated with the formation of hyphae over blastoconidia, including: low oxygen tension, high temperature and impoverished growth media (75, 135). The ability of *C. albicans* blastoconidia to form the outgrowth of a hypha, commonly called a germinal root, at 37°C in the presence of serum is a standard rapid laboratory test for identification (198).

C. albicans can also form larger (diameter 8 to 12 μM), rounded cells called chlamydospores at the terminal ends of hyphal segments (135).

Chlamydospores can develop after growth on nutrient deficient media and can be useful for species identification (127, 198). The physiological status of the chlamydospore is not certain because there are no credible reports of its germination (75). Since chlamydospores have only rarely been observed in host tissues (84) and the structures are much more fragile compared to yeast cells (73) they are not regarded as an adaptation to survival but rather an *in vitro* form of *C. albicans*.

I B. Human Commensal

Candida are found on plants and as part of the normal flora in the alimentary tracts of mammals (135). *Candida* species grow commensally in the human gastrointestinal tract and on the mucous membranes of the oropharynx and genitourinary tract (61, 135). The overall carriage rate of *Candida* species in the gastrointestinal track of healthy individuals has been estimated to be at least 80% (198). *C. albicans* is the most commonly isolated *Candida* species, comprising 50 to 70% of all such isolates (198). *C. albicans* can also be isolated from the oral cavity of up to 50% of young adults (135) and from the vagina of 20% of women (63). The high prevalence of *C. albicans* in the healthy

population likely contributes to this species also being the most common cause of symptomatic superficial and systemic fungal infections (162). There are five species of *Candida* that are important human pathogens and which are encountered with higher frequency in clinical practice, these include: *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. glabrata* (52, 179). *C. dublinensis* and *C. lusitaniae* are also isolated, but less frequently.

II. Opportunistic Human Pathogen

II A. Superficial Infections

Candida is an opportunistic pathogen and may be associated with pathological conditions in a patient whose anatomical integrity, normal bacterial flora or immune system has been impaired by disease or iatrogenic intervention (Table 1.1). *Candida* can also cause infections like vaginitis in the presumably normal human host. *Candida* species are common pathogens in the immunocompromised patient, the postoperative patient, the low birth weight neonate, and in patients requiring advanced technology for life support (40).

Readily diagnosed and easily treated clinical forms of superficial candidiasis include infections of the skin, nails, and mouth. Often, these patients have some underlying factor that promotes a superficial *Candida* infection. For example, any process causing skin maceration in healthy individuals will increase the susceptibility of the involved site to *Candida* invasion (52). Hippocrates (86) first described oral candidiasis as an infection of the debilitated in the fourth century BC. *Candida* infection of the oral cavity affects approximately 5% of normal newborns (190). Infection of the esophagus by *Candida* is considered a defining illness for patients with AIDS (142). The development of oral candidiasis in HIV-infected patients often indicates a drop in the $CD4^+$ lymphocyte count to $200/mm^3$ or less (179).

Local inoculations of *Candida* can also cause infections of the esophagus, gastrointestinal tract, genital-urinary tract, and respiratory tract. Mucocutaneous forms of *Candida* infection are often related to defects in cell-mediated immunity, while systemic spread is generally associated with neutropenia (61). Recurrent and chronic vaginitis is a recognized but poorly understood problem with no definitive cure and believed to be caused by some immunological deficiency. Chronic mucocutaneous candidiasis is a collection of syndromes in which patients have chronic and/or recurrent infections of the skin, nails, and

mucous membranes with *Candida* species (100). Chronic mucocutaneous candidiasis is a rare disease that presents mostly in children and adolescents. Most patients have a negative delayed-type hypersensitive skin reaction against *C. albicans* and decreased lymphokine production after stimulation by *Candida* antigens (29). Patients with chronic mucocutaneous candidiasis require prolonged therapy and relapses are frequently seen when therapy is stopped (161).

II B. Systemic Infections

The gastrointestinal tract is commonly believed to be a major portal of entry to the intravascular compartment (43). When introduced into the bloodstream, *Candida* may produce a life-threatening and progressive systemic disease referred to as disseminated candidiasis. The spectrum of hematogenously disseminated infection is broad and any organ or combination of organs can be infected acutely or chronically (24). Widespread dissemination commonly occurs to the: liver, kidneys, spleen, lung, heart, brain and bone (52, 130, 108). Several important predisposing factors to candidemia include diabetes

mellitus, renal failure, burns, hematological malignancies, neutropenia, cytotoxic chemotherapy, corticosteroids, organ transplantation, broad spectrum antibiotics, hyperalimentation, and indwelling intravascular catheters (202, 205) (Table 1.1).

III. Epidemiology of an 'An Emerging Threat'

C. albicans was identified in the 1992 Institute of Medicine report as an important emerging pathogen (defined as a disease of infectious origin whose incidence in humans has increased within the past two decades or whose incidence threatens to increase in the near future) (45). *Candida* is the fourth most common cause of nosocomial bloodstream infection in the United States (10, 31) and the rate of primary blood stream infections continue to increase (12, 96). In the Americas, *C. albicans* is the species most frequently isolated from the bloodstream (139). The dramatic increase in the incidence of *Candida* infections is likely related to the increase in the immunosuppressed population in the hospitals and community (45). Candidemia has been shown to occur in 11 to 27% of patients with prolonged neutropenia from bone marrow transplantation or leukemia (117). Mortality in these

infected neutropenic cases can be as high as 95% (117). In another study of liver transplant recipients, invasive fungal infections occurred in over 20% of patients within 100 days of transplantation and *Candida* accounted for 82% of all infections (39).

The morbidity and mortality associated with systemic infections caused by *C. albicans* remains unacceptably high because of difficulties in diagnosis and the significant toxicity that accompanies treatment (58). The attributable mortality rate is 38% in the United States (184) and between 19 and 23% in Canada (96, 181, 204). A study of postoperative patients has shown that candidemia increases the overall mortality to 83%. (174). In this aforementioned study, all cases of candidemia left untreated for more than one day were fatal (174). Candidemia also prolongs the length of hospital stay by 30 days above that required for the treatment of the surviving patients' underlying conditions (201). Considering the mortality with the reported rate of nosocomial bloodstream infections being caused by *C. albicans*, the incidence of candidiasis represents a serious patient management problem.

IV. Difficulties with Diagnosing Candidemia

The early diagnosis and treatment of disseminated candidiasis is important for a good prognosis (53). Diagnosis of disseminated candidiasis is often too late to be of any benefit to the patient. In one study, only 15 to 40% of patients with candidemia were diagnosed early enough to receive effective antifungal treatment (42). The diagnosis of disseminated candidiasis is usually difficult to establish because the clinical symptoms are generally nonspecific (117, 53). Candidemia is often suspected if a patient has an unrelenting fever and remains unresponsive to broad spectrum antibiotics (14). Confirmatory diagnosis has been traditionally dependent on the isolation and culture of the fungus. However, laboratory diagnosis is complicated by the commensal existence of *Candida* in humans. One study suggests that up to 50% of *Candida* positive blood cultures are due to transitory or self-limiting candidemia (113, 54). For example, indwelling vascular catheters are often colonized and prompt removal eliminates the organism from the bloodstream (205).

Lengthy incubation times and poor sensitivity further complicate positive cultures of blood from patients with candidemia. The culture of *Candida* from the blood of patients is often negative and has been

shown to be positive in no more than 50% of neutropenic patients with disseminated candidiasis (49). In one study, blood cultures were positive in only 35% of patients with disseminated candidiasis (117). The detection of candidemia requires a good blood culture system and commercial manual and automated systems are in use. The automated commercial systems employ a rich culture medium and rely on the detection of *Candida* in the blood by measuring carbon dioxide, charged radicals or redox potential (119, 148). These systems have improved isolation rates but many patients still have negative cultures.

At autopsy, multiple organ *Candida* abscesses routinely fail to propagate viable fungus (18). Laboratory diagnosis of disseminated candidiasis relies on deep tissue biopsy for *Candida* detection. However, clinicians are reluctant to undertake this invasive procedure in immunocompromised patients already predisposed to systemic disease. These patients often have low platelet counts due to chemotherapy or other interventions. Research into improvements in the diagnosis of candidemia has been focused on nonculture-based detection methods (155). Previous methods have relied on the detection of *Candida* antigens and antibodies (78, 113). Further developments in detection were based on the detection of nonantigenic *Candida* components such as D-arabinitol, mannose and (1→3)-beta-D-glucan (104, 196).

Currently, PCR based assays are being evaluated for the detection of *Candida* DNA (35, 129).

V A. Systemic Antifungal Therapy

Progress in antifungal therapy has been slow and only a limited number of antifungal agents are available for the treatment of systemic fungal infections (58). Until recently, limited resources and effort were expended by the pharmaceutical industry to develop new antifungal agents because fungal infections were not considered a major health problem. In addition, the development of antifungal agents has been complicated by the shared cell biology between mammalian and fungal cells (120). Effective antimicrobial therapy relies on selective toxicity and requires that the antimicrobial agent reach the site of infection in sufficient concentration to inhibit or kill the infecting microorganism with minimal toxicity to the host (5). Antifungal agents that damage or kill fungal cells are likely to have toxic effects on mammalian cells as well. Antifungal agents currently used to treat systemic mycoses are limited to three major groups: the polyene agents (e.g. amphotericin B), the azole agents (e.g. fluconazole), and 5-fluorocytosine (Figure 1.1). The

echinocandins are a fourth class of systemic antifungal agent that are in development (56). The echinocandins inhibit the beta-1,3-D-glucan synthase enzyme complex that forms glucan polymers in the cell wall of yeasts.

V B. Amphotericin B

Amphotericin B was first approved in 1957 and still remains the most broad spectrum antifungal agent (198). Amphotericin B is the agent of first choice for the treatment of serious systemic fungal infections (46). Amphotericin B is considered fungicidal and has a minimal risk of resistance development (185). Amphotericin B is an amphoteric polyene molecule, containing both a hydrophobic segment of double bonds and a hydroxylated hydrophilic segment (Figure 1.1). The agent exerts its antifungal effect through the hydrophobic binding to sterol compounds in the fungal cell membrane. This binding alters the fungal cell membrane permeability and causes leakage of cytoplasmic constituents (67, 121). It has been proposed that the interaction of amphotericin B with ergosterol results in the production of aqueous pores consisting of amphotericin B molecules linked hydrophobically to

ergosterol with the hydroxyl residues facing inward (91). Subsequent to binding to ergosterol, amphotericin B has also been shown to undergo autooxidation with the production of reactive intermediates that cause oxidative damage to the cell (172, 173).

Amphotericin B has a much higher affinity for ergosterol-containing membranes of fungal cells than for the cholesterol-containing membranes of mammalian cells (27, 67, 154). The toxicity of amphotericin B in humans is likely due to cell damage caused by the agent binding to cell membrane cholesterol within host tissues (98). Amphotericin B is bound to tissues in the highest concentrations in the liver and spleen and in lesser amounts in the kidney and lung (108). Amphotericin B is one of the most toxic antimicrobial agents in clinical use today (25). The toxicities are so high that they frequently determine the dose and schedule of agent administration instead of the response to therapy (25). Nephrotoxicity is the most serious side effect of amphotericin B therapy and most patients develop some manifestation of renal damage (81). Chills, rigors, fever, hematologic effects and thrombophlebitis are also common side effects (70). The toxicity seen in adults is less in neonates and children (111, 161, 168).

Amphotericin B has also been formulated in liposomes (Ambisome) (2), lipid complexes (Abelcet) (152), and colloidal

suspensions (Amphotec) (92, 203) to reduce the toxicity of the parent compound and permit higher dosing. The recommended dose for the parent compound to treat disseminated candidiasis is 0.5 to 1 mg/kg/day (46). Higher dosages of the lipid-based amphotericin B have been administered safely (46). The effective dose may vary from one formulation to another and is approximately 3 to 5 mg/kg/day for the lipid based formulations (50). However, in animal models of infection, higher doses for the liposomal preparation of amphotericin B on a per kilogram basis was generally required to achieve the same antifungal effect of the parent compound (137). Considerable differences exist between the pharmacokinetic profile of amphotericin B and the lipid-based preparations, including tissue distribution (199). Liposomes allow for the preferential deposition of amphotericin B in areas with large numbers of reticuloendothelial cells such as the spleen, liver and lungs (36). The early results from clinical trials indicate that the lipid formulations may offer a therapeutic improvement over the parent compound with lower nephrotoxicity (80, 153, 185). At present, the efficacies of the various lipid formulations of amphotericin B for the treatment of disseminated candidiasis have not yet been directly compared with the parent compound. Thus, it has yet to be convincingly demonstrated that the lipid formulations of amphotericin B improve the outcome of infection

compared to the parent compound (58, 41). The cost of the lipid formulations are a major factor limiting prescription in many institutions, where use is often restricted to patients that are refractory to, or intolerant of, amphotericin B (50).

Although amphotericin B has been available for over 40 years, its pharmacokinetics are still poorly understood and no breakpoints have been defined (7, 160). Peak serum levels of 0.5 to 2 μg/mL of amphotericin B are described as being typically achieved after initial intravenous infusion of 1 to 5 mg/kg/day (50 to 70 mg/day typically). (17, 79, 108, 160). This peak serum concentration falls rapidly and then slowly reaches a plateau between 0.2 and 1 μg/mL (14, 169). The average steady-state concentration of amphotericin B in the plasma has been described to be about 0.5 μg/mL (160), and it suggested that this concentration might represent the upper end of the treatable range.

V C. Azoles

The azole class of antifungal agents was introduced in the late 1960s but only four agents have been approved for the treatment of systemic fungal diseases: miconazole, ketoconazole, fluconazole, and itraconazole (58). A major concern with the azole antifungal agents is

the potential for hepatic toxicity. The azoles are divided into two groups, imidazoles and triazoles, based on the number of nitrogen atoms in the five-membered ring structure (Figure 1.1) (120). Imidazoles contain two nitrogen atoms with a complex side chain attached to one of the nitrogen atoms. The imidazoles that were previously used for systemic therapy are miconazole and ketoconazole. Miconazole was the first agent to be administered intravenously for the therapy of systemic fungal infections and ketoconazole is an orally absorbed imidazole (44). Miconazole has solubility problems and cardiorespiratory toxicity (77). Ketoconazole is unreliable due to uncertain oral bioavailability.

The triazoles are similar structures containing three nitrogen atoms in the five-membered ring. The triazoles currently indicated for systemic therapy are fluconazole and itraconazole. Itraconazole and fluconazole have less potential for toxicity than the imidazoles because of their higher specificity of binding to the fungal cell cytochromes (59). The orally active triazoles have superseded the older imidazoles due to their superior efficacy and lower toxicity (41). Itraconazole has a less optimal pharmacokinetic profile compared to fluconazole. Although all of the azole antifungal agents are active against *Candida* infections, fluconazole has been shown to be the most useful in treating of hematogenously disseminated infection (106). Ketoconazole may also

have limited efficacy against *C. tropicalis* and *C. krusei*, the latter of which is inherently resistant to fluconazole (44).

Several new azole derivatives have been developed with enhanced spectra of activity and improved pharmacokinetics (88). Voriconaozle is a new triazole structurally related to fluconazole (90). It has an improved efficacy (90) and is more potent in inhibiting ergosterol synthesis (165), and has enhanced activity against *Aspergillus* species. Posaconazole is structurally related to itraconazole and has comparable activity to voriconazole against several *Candida* species but with a longer half-life (30). Ravuconazole is less structurally related to the other azole antifungals but exhibits high *in vitro* activity against most *Candida* species (138). No clinical trial data is presently available for ravuconazole.

The azole antifungal agents are fungistatic and thus only inhibitory. The principle mechanism of action of the azole compounds in fungal cells, is the inhibition of cytochrome P-450 enzymes (92). The major enzyme inhibited is the 14alpha-demethylase that is responsible for the conversion of lanosterol to ergosterol. The inhibition of ergosterol synthesis and depletion of ergosterol in the fungal cell membrane leads to the production of a defective cell membrane with altered permeability (149). The imidazole agents may also have an

effect on the fatty acids of cell membranes, causing leakage of intracellular cations, proteins, amino acids and nucleotides (193).

V D. Flucytosine

Flucytosine (5-fluorocytosine) is a pyrimidine base cytosine analog that functions as a competitive antimetabolite for uracil in the synthesis of yeast RNA (195). Cytosine permease actively transports flucytosine across the fungal membrane into the fungal cell. The agent is deaminated by cytosine deaminase to 5-fluorouracil selectively in the fungal cell and incorporated into RNA. The specificity of cytosine deaminase accounts for the spectrum of activity of flucytosine against fungi and its low toxicity to the mammalian host. Fungi that lack cytosine deaminase are not susceptible to flucytosine, because the parent compound is itself inactive. Flucytosine is also converted to 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase and DNA synthesis (143).

About 5% to 10% of *Candida* species strains are inherently resistant to flucytosine and 5% develop resistance during therapy (11). These strains of *Candida* that are resistant to flucytosine are predominantly of serotype B. Consequently, flucytosine is indicated only

as combination therapy with amphotericin B for serious *Candida* infections. *In vitro* studies have also demonstrated synergistic interactions between flucytosine and amphotericin B (128).

VI. *In vitro* Antifungal Susceptibility Testing

VI A. Rationale

In vitro susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the development of primary or secondary resistance in clinical isolates (141, 160). The rationale and design of *in vitro* antifungal susceptibility testing are similar to those currently used for testing antibacterial agents (141). *In vitro* susceptibility tests are meant to provide: (i) a correlation between *in vivo* activity and therapeutic outcome, (ii) a measure of the relative activities of two or more antifungal agents, and (iii) a means to monitor the development of drug resistance (69, 134).

It must also be recognized that there are inherent limitations with *in vitro* susceptibility testing because of the artificial nature in the

interaction between the microorganisms and antifungal agents (5). Susceptibility testing does not reproduce *in vivo* conditions, in part, because: (i) the host defense is absent, (ii) the agent concentration is essentially static, and (iii) the test inoculum size is probably at variance with the infectious load (4). The determination of *in vivo* efficacy of antifungal agents thus takes into account both host defences and intrinsic antimicrobial activity (26). Although, it may not always be possible to identify which patients will respond to antifungal therapy, *in vitro* antifungal susceptibility testing can help identify those patients who will have a decreased probability of responding (159).

VI B. Testing Methods

Antimicrobial susceptibility testing was first initiated by Alexander Fleming in the 1920's (5). In this first assay, Fleming placed penicillin extracts into ditches cut into agar and cross-streaked staphylococci and streptococci (62). The methods that have been employed for use in antifungal susceptibility testing include broth dilution (macrodilution and microdilution), and agar-based testing methods (58). Disk-based susceptibility testing is both convenient and economical (160) and has been shown to have good correlation with the reference broth method

for fluconazole susceptibility testing (107, 122, 194). The commercial E-test makes use of a calibrated strip impregnated with a gradient of antifungal agents placed on an agar plate lawned with the fungal strain (160). The E-test has good correlation with reference broth method susceptibility testing of most *Candida* species with the azoles and amphotericin (140, 145)

In 1997 the National Committee for Clinical Laboratory Standards (131) Subcommittee on Antifungal Susceptibility Testing published a reference method for broth dilution susceptibility testing of yeast cells (131). The guidelines were developed to allow for the development of standard breakpoints to guide therapy and to decrease interlaboratory variability. Initially, the M27-A protocol recommended the use of a broth macrodilution method, but a simpler broth microdilution method was determined to produce comparable results (57). This reference protocol was the result of a series of collaborative studies that focused on some of the testing variables. *In vitro* antifungal susceptibility testing and endpoint determination is influenced by a number of technical variables, including medium composition, inoculum size, duration and temperature of incubation (48, 59, 69, 134). With respect to the parameters of this dissertation, the primary issue of

consideration with regards to antifungal susceptibility testing was the improvement of the speed and objectivity of endpoint determination.

VI C. Endpoint Determination

Susceptibility testing data are expressed as agent concentrations (μg/mL) and represent minimum inhibitory concentrations (MIC). The MIC value is based upon a predetermined endpoint and is the concentration of antifungal agent that inhibits the growth of a fungal strain under standardized conditions. Visual inspection, turbidity and optical density measurements, colourimetric changes, release of labeled carbon dioxide, determination of adenosine triphosphate, dry weight determinations, and other methods have been used to measure the effect of different concentrations of an antifungal agent upon fungal growth (123, 144, 150). Traditionally, the MIC was considered the lowest concentration of an antifungal agent that completely inhibited growth of the fungi, as detected visually (58). Using the less stringent criterion for the MIC endpoint, a prominent decrease in turbidity, has increased interlaboratory reproducibility for flucytosine and the azoles (57).

VI D. Difficulties with Susceptibility Testing of *Candida*

The broth microdilution method for antifungal susceptibility testing has been extensively evaluated for *Candida* species, however the clinical interpretation of susceptibility testing remains somewhat controversial (101). *In vitro* susceptibility testing should give a reliable prediction of *in vivo* response in human infections. Unfortunately, the evidence for clinical correlation with *in vitro* test results is limited at best (141). The most useful correlation between susceptibility testing and outcome is found in HIV-infected patients with oropharyngeal candidiasis being treated with azole antifungals (132).

There remains uncertainty over the optimal reading of MIC end-points and the duration of incubation for antifungal susceptibility testing (89). The partial inhibition or "trailing effect" that is commonly observed with the azole agents precludes the determination of well-defined endpoints and creates a great deal of variability and confusion (58). For some strains of *Candida* species, persistent growth occurs even in the presence of high concentrations of azoles (114). A paradoxical effect with the azoles has also been observed, whereby growth increases with increasing concentrations of antifungal agent (175). The paradoxical or 'eagle' effect has also been observed with the semi-synthetic lipopeptide

cilofungin (156). This *in vitro* effect is thought to be due to either the aggregation of the drug at high concentrations that prevents its normal inhibitory effect or to culture medium effects (156).

In addition, some *C. albicans* isolates do not show the "trailing effect" after 24 hours of incubation, but do after 48 hours (114, 157, 186). Isolates have been termed to have a low-high phenotype when the strain is susceptible after 24 hours of incubation (fluconazole MICs < 8 μg/mL) and appears resistant after 48 hours (fluconazole MICs > 64 μg/mL). In general, the clinical correlation with the *in vitro* susceptibility tests results for azole antifungal agents have been quite poor (48, 66, 58).

Establishing a clear correlation between amphotericin B MICs and outcome has been difficult (160). Using the approved NCCLS methodology (131), susceptibility testing of *C. albicans* with amphotericin B produces a narrow range of MIC results for most isolates (160), in the range of 0.25 to 1.0 μg/mL (131). This narrow range of MICs makes detecting differences in susceptibilities among isolates extremely difficult. Most *Candida* species isolates are inhibited by ≤1.0 μg/mL of amphotericin B and as a consequence a MIC of greater than 1.0 μg/mL may represent resistance but no breakpoints for amphotericin B currently exist (131, 132, 160). Clinical trials have disclosed that there

is only a marginal correlation between amphotericin B MICs and clinical outcome (158). However, *in vivo* resistance of amphotericin B does exist (160). Studies (28, 147) have found that patients with candidemia with MICs to amphotericin B greater than 0.8 μg/mL were significantly more likely to die because of that infection than patients with infection due to *Candida* species with MICs to amphotericin B at 0.8 μg/mL or less. A prospective study by Nguyen *et al.* (133) that correlated *in vitro* amphotericin B susceptibility results with the outcome for 105 patients with candidemia, showed that the minimal lethal concentration (MLC) range of amphotericin B was significantly broader than the MIC range and that the MLC was a better predictor of *in vivo* resistance. The MIC was defined as the lowest concentration of amphotericin B that completely inhibited growth at 48 h (131) and the MLC was defined as the lowest concentration of agent from which the subcultures yielded less than five colonies (5). Microbiologic failure was defined as either persistence of *Candida* in the bloodstream despite greater than three days of amphotericin B therapy or development of breakthrough *Candida* fungemia while receiving amphotericin B for at least three days for either prophylactic or empiric indications (133).

PART TWO: Viability, Killing and Injury

VII. Replication Competency

Difficulties in quantifying microbial killing are due in large part to the properties that are attributed to the state of being alive, because the presence of dead microbes are inferred retrospectively from the estimates of these properties (118). The enumeration of CFUs on solid media was first described in 1881 (103) and is still considered to be the 'gold standard' criterion for determining microbial viability (94, 118). Consequently, microbial cell death is defined as the irreversible loss of the ability to grow and divide and the empirical test of death is the culture of cells on solid media whereby a cell is considered dead if it fails to produce a colony on solid media. (93). However, it is also well recognized that only a fraction of viable microorganisms replicate when stressed (34, 99, 118) and a failure to replicate may be due to many deficiencies other than cell death (95). Consequently the terms mitotic viability (37), reproductive viability (85), and replication competency (114) have been used in place of the more commonly used terms viable count or viability, when enumerating fungal cells using the agar plate technique (125, 191). Yeast cells will remain unchanged in cell size if

they are unable to increase in size and proceed past a certain point in the division cycle (3).

VIII. Cellular Integrity and Metabolic Potential

Several methods that evaluate membrane integrity and metabolic activity have shown that the number of viable fungal cells estimated using the agar plate technique to be underestimated. These methods have included: the direct microscopic proliferation assay (87, 151); vital staining with Giemsa (64), Methylene blue (72) and acridine orange (170); tetrazolium salt reduction (112, 182, 188); and flow cytometry using tetrabromofluorescein (38) and fluorescein diacetate (20). It has been suggested that intact cells that no longer have the ability to form colonies may retain some metabolic activity and that different viability assays are measuring different fractions of the total population (95).

Lehrer *et al.* reported consistently higher viability of *C. albicans* using a dye exclusion method compared to the agar plate method, and suggested that the two assays were measuring different characteristics of *C. albicans* (110). The same result was obtained by Levitz *et al.* using a (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) (MTT)

tetrazolium reduction assay that measures mitochondrial dehydrogenase activity (112). The electron transport system is mediated by the action of several dehydrogenase enzymes and direct measurement of dehydrogenase enzyme activity has been shown to be a reliable indication of metabolic activity (136, 180, 187). The MTT tetrazolium assay has also consistently produced significantly higher values for viability than the agar plate method for *C. albicans* undergoing prolonged incubation in distilled water (87). The results of the MTT assay were shown to correlate well with acridine orange uptake and the microscopic observation of cell division (87).

The reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to formazan in mitochondria has also been compared with colony formation using the agar plate method and DNA repair capacity in *Saccharomyces cerevisiae* cells treated with ultraviolet irradiation (37). Before repair of ultraviolet photoproducts (cis-syn cyclobutane pyrimidine dimers) was complete, these two assays produced very different results, implying that the damaged cells were metabolically competent but were unable to replicate. These results indicated that tetrazolium reduction in UV-irradiated, nondividing yeast cells was a much better indicator of cell survival than replication competency.

When evaluating cells in the same physiological state, the concentration of ATP in cells has been shown to be a direct indicator of the number of viable cells (126, 167), because ATP is rapidly destroyed when cells die (116). However the concentration of ATP in yeast varies greatly depending on the growth rate (32, 200) and cells can utilize ATP in the complete absence of growth (163). In addition, the ATP level required for maintaining yeast viability is only a minor proportion of the ATP consumed in nonbiosynthetic functions during growth (109). In bacteria, low levels of ATP have also been associated with actively metabolizing bacteria with inhibited replication (33). An obvious limitation of using metabolic activity as a determinant of viability is that inactive cells with very little metabolic activity could appear to be dead.

IX. Loss of Replication and Recovery from Sublethal Stress

Lloyd *et al.* have suggested that an equilibrium exists in normally dividing microorganisms between the energized and de-energized state, whereby small disturbances are tolerated but extreme stress reduces viability to the point of cell death or to a state where normal cellular activities cease to function (115). For a cell to survive only a very small

part of its metabolic potential needs to be expressed. These have been collectively referred to as maintenance functions (109), and include: maintenance of osmotic potential, turnover of essential cell materials, and maintenance of the membrane potential. It is hypothesized that if the energy for these processes is not provided the cell will irreversibly cease to function (118). Thus it has been postulated that fungal cell death is a gradual process and that the loss of the ability to replicate may be an initial event to the onset of eventual cell death (95). Included in this definition is the concept that sublethally stressed fungal cells have the capacity to overcome some physiological damage and to recover from injury (15, 189). Abbott *et al.* have shown that miconazole-treated *C. albicans,* unable to replicate on drug-free media, can undergo a restoration of ATP levels with 10 mg/mL of glucose (1).

Sublethally stressed fungal cells exhibit a lag before the onset of replicative growth as they repair cell damage (15, 23, 60, 71, 124). The recovery process has been shown to take longer on agar surfaces, compared with liquid broth, and preincubation in broth is sometimes required (23). In addition, the media composition required for recovery of injured microorganisms may be different from that used for optimal growth under normal conditions (146). The length of recovery time for

injured fungal cells has also been empirically shown to decrease with temperatures below the growth optimum (16, 65, 166, 97).

X. *C. albicans* Replication Competency and Viability – Observations in Clinical Microbiology

There are a number of clinical situations where *C. albicans* has been shown to exist in a state of very slow or nonexistent replication. The *C. albicans* cells present in these conditions are also refractory to killing with amphotericin B treatment. The tetrazolium reduction assay was used to evaluate *C. albicans* viability in the biofilm mode of growth because the plate count method was a poor estimation (82). These *C. albicans* cells were shown to be resistant to amphotericin B irrespective of the morphological form (8, 9, 83). It was suggested that the resistance mechanism may be due to the slow growth rate of *C. albicans* in the biofilm growth mode and the reduced antifungal penetration into the biofilm (8, 21, 177). The increased amphotericin B resistance observed with sessile *C. albicans* cells from biofilms also occurs with planktonic *C. albicans* at low rates of growth (68).

Similar to the microorganisms in experimental endocarditis (51, 55), the subcutaneous *C. albicans* abscesses in a murine model have been shown to be relatively dormant with a reduced capacity to form colony forming units on agar media (171). Also, the biopsy of infected tissue from patients with hepatosplenic candidiasis usually reveals *Candida albicans* (24), but the organism is cultured from only 30% of tissue specimens and blood cultures are uniformly negative (24, 183). These patients generally do not respond to amphotericin B therapy and in many cases infection can persist despite administering amphotericin B for six months to total doses of as high as 5 g (24). It has been suggested that the failure to respond to amphotericin B therapy may be due to the pharmacokinetic properties of this agent, such as poor penetration into the infected areas (160). However, Thaler *et al.* observed that cultures are frequently negative, especially in patients who have been pretreated with amphotericin B (183). Thaler *et al.* also reproduced this effect in an animal system (183). *C. albicans* could not be cultured from the liver lesions in a neutropenic rabbit model if the animals were treated with amphotericin before death.

Microbial physiology is an important determinant for the effectiveness of antimicrobial agents. The specific physiologic state of the fungal cell, particularly the rate of cell replication, directly influences the activity of

antifungal agents (21, 22). The use of antimicrobial agents with mechanisms of action that interfere with the different growth phases or altered physiological states that microbes use for survival, can provide a approach to antimicrobial therapy that can be best described as creating physiological conflict for the infecting microorganism (178). When these agents are used in combination for therapy, their use creates physiological conflict for the microorganism when it enters an altered physiological state that can result in enhanced killing (178).

XI. RATIONALE AND HYPOTHESIS

This work describes the use of several assays that were developed with the aim of changing how viability in *C. albicans* is determined. Instead of using visual estimates of growth and CFUs, we proposed a more global view using specific assays that separately measured attributes ascribed directly to vitality and mortality. I conclude that this type of analysis could provide profound insights since some of the most important work in microbiology involves research designed to improve the killing of pathogens. I chose to test this mode of thinking in

an increasingly important field of clinical microbiology, mycology, with one of its most important pathogens, *C. albicans.*

The morbidity and mortality due to invasive infections caused by *C. albicans* remains unacceptably high due to difficulties in diagnosis and treatment. The dramatic increase in the rate of nosocomial infection by *C. albicans* and the large number of antifungal agents that are being developed in response to this growing problem indicate that antifungal susceptibility testing will be of even greater importance in the near future. Antifungal susceptibility testing remains dependent on long incubations with semiquantitative and subjective endpoints. The drug of choice for the treatment of invasive candidiasis and the standard by which new antifungal agents are judged is amphotericin B. Treatment with amphotericin B is often prolonged and refractory. Significant controversy exists about the correlation between clinical outcome and amphotericin B susceptibility testing. Fluconazole is less toxic than amphotericin B and is used as an alternative in some circumstances. However, endpoint determination for the azole antifungal agents is problematic and a major source of interlaboratory variability.

To address these difficulties, a number of assays were first developed and optimized to evaluate *C. albicans* viability. These tools were then applied to *C. albicans* killing using antifungal susceptibility

testing. The following chapters discuss the successes we have had at improving the accuracy and speed of endpoint determinations utilizing these techniques. Moreover, by challenging the traditional views of fungicidal killing, we discuss the development of a new insight into the action of amphotericin B and describe a novel physiological state whereby *C. albicans* can survive amphotericin B treatment and maintain a capacity for resuscitation of growth.

XII. TABLES

Table 1.1. Conditions and therapies predisposing individuals to invasive *Candida* infections

CONDITIONS
• Granulocytopenia
• Advanced HIV infection
• Bone marrow transplantation
• Solid organ transplantation (immunosuppressive agents)
• Very low birth weight (<1500 g)
• Diabetes mellitus
• Fibrotic and cavitary lung disease
• Severe burns or trauma
• Severe malnutrition or debilitation
• Intravenous drug abuse (nonsterile equipment)
• Hormone imbalance
THERAPIES
• Intravenous hyperalimentation
• Broad spectrum antibiotics
• Indwelling catheters and devices
• Prosthetic devices (heart valves, shunts)
• Corticosteroid treatment
• Hemodialysis and peritoneal dialysis

XIII. FIGURES

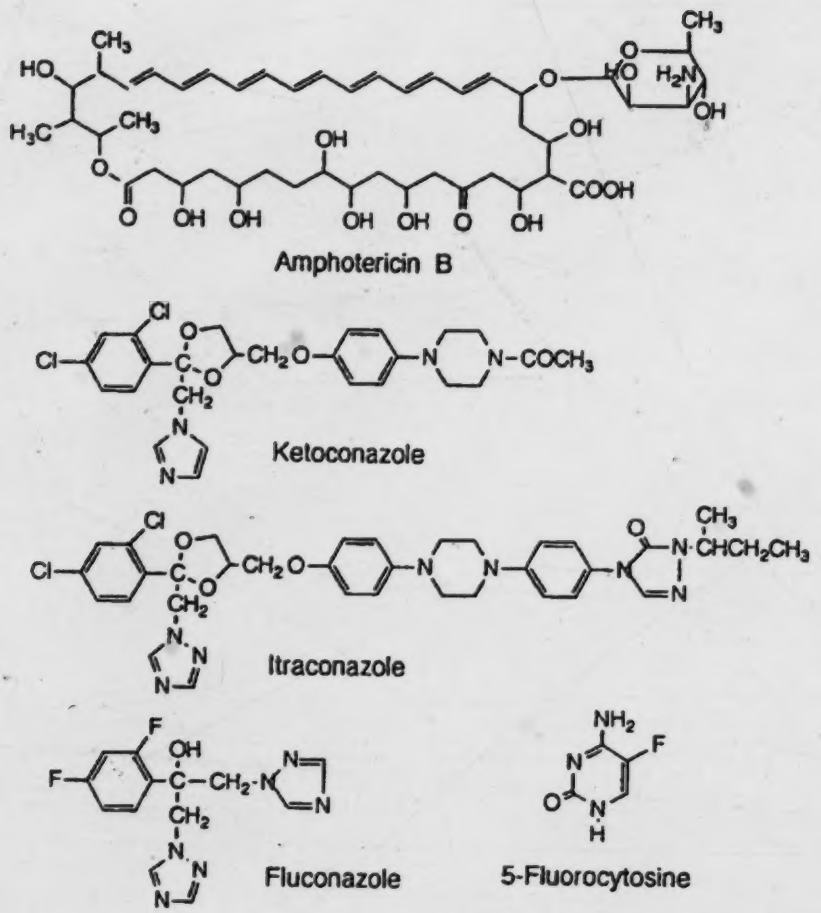

Figure 1.1. Structures of systemic antifungal agents.

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Chapter 2

A Novel Fluorescent Broth Microdilution Method for Fluconazole Susceptibility Testing of *Candida* species

Adapted from the original publication:

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I. INTRODUCTION

Candida is the fourth most common cause of nosocomial bloodstream infection in the United States (2, 4) and the rate of primary blood stream infections continue to increase (3, 9). In the Americas, *C. albicans* is the species most frequently isolated from the bloodstream (16). Candidemia is often difficult to diagnose and refractory to therapy. The attributable mortality rate is reported to be 38% in the United States (28) and can be estimated to be between 19 and 23% in Canada after comparing the mortality rate at 3 large medical centres (9, 25, 29).

Use of the broad spectrum antifungal fluconazole for the treating of serious systemic *Candida* infections has increased. Fluconazole is a less toxic alternative to amphotericin B and has been recommended as primary therapy for candidemia in nonneutropenic and stable neutropenic patients who have not received prior fluconazole treatment and in whom *C. krusei* is unlikely (5, 24). *In vitro* susceptibility testing for fluconazole is of clinical importance, as therapeutic success depends substantially on achieving plasma levels that are sufficiently higher than MIC values (22).

Despite great advances in standardizing antifungal susceptibility testing, azole endpoint determination continues to be problematic,

subjective, and a major source of interlaboratory variability (6, 18, 23). The trailing growth phenomenon is often responsible for these difficulties, whereby partial inhibition of fungal growth occurs over the range of azole concentrations (6, 13, 14, 23).

Previous work has demonstrated the utility of using fluorescent dyes to assess the viability of *C. albicans* exposed to amphotericin B (10). We have investigated the use of the vitality-specific dye carboxyfluorescein diacetate (CFDA) in the standardized NCCLS M27-A broth microdilution method (12) to assess susceptibility of *Candida* spp. to fluconazole. In this study we compared the NCCLS microdilution method with a CFDA modification in determining fluconazole susceptibility for common clinical yeast isolates (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*).

II. MATERIALS and METHODS

Yeast isolates. Yeast isolates (Appendix I) were obtained from the National Centre for Mycology, Division of Microbiology and Public Health, Edmonton, Alberta, Canada; and two low-high phenotype *C. albicans* strains were kindly supplied by John H. Rex from the Center for the Study of Emerging Pathogens and Reemerging Pathogens,

Laboratory of Mycology Research, University of Texas Medical School, Houston, Tex. The identity of the isolates was confirmed by standard methods (26), isolates were stored in skim milk at –70°C and then subcultured twice on Sabouraud dextrose agar (Difco, Sparks, MD) before use. These strains included: 48 clinical isolates of *C. albicans* and homologous control strains ATCC 90028, ATCC 90029, ATCC 24433, ATCC 10231, ATCC 20408; 4 clinical isolates of *C. tropicalis* and ATCC 750; 4 clinical isolates of *C. glabrata* and ATCC 90030; and 4 clinical isolates of *C. parapsilosis* and ATCC 22019.

Antifungal susceptibility testing. The reference NCCLS broth microdilution method was performed as described in the M27-A document (12). Fluconazole concentrations were diluted in RPMI 1640 medium with L-glutamine, and morpholinepropanesulfonic acid (MOPS) buffer at 165 mM and pH 7 (Angus Buffers & Biochemicals, Niagara Falls, NY) and 100 μL aliquots were placed into the wells of 96-well microtiter Linbro plates (Flow Laboratories Inc., McLean, VA) with clear, U-shaped well bottoms (Appendix I). The final concentrations of fluconazole ranged from 0 to 64 μg/ml. Six *C. albicans* strains were tested per 96-well plate, which allowed for the empty outermost wells to be filled with sterile water to minimize evaporation.

Five *C. albicans* colonies ≥1 mm diameter were suspended in sterile 0.85% saline and adjusted to a final concentration (after inoculation) of 0.5-2.5 x 10^3 cells per ml in RPMI 1640-MOPS medium. The inoculum was added to the fluconazole trays and incubated at 35°C and evaluated at 24 and 48 h.

(i) Reference MIC endpoint. The reference broth microdilution was scored by visually comparing the growth in each well with that in the growth control (drug-free) well. The MIC was defined as the minimum drug concentration at which visual growth was determined to be 80% relative to that of the growth control (12).

(ii) Fluorescent MIC endpoint. The CFDA-modified microdilution method was identical to the method described for broth microdilution susceptibility testing, except that after determination of visual endpoints at 24 and 48 h incubation at 35°C a fluorescence assay was also performed. The supernatant solutions were first removed from the tray wells using a multichannel pipettor and the remaining fungi were suspended to 200 μL per well in 35°C-warmed 0.1 M MOPS buffer at pH 3.5 with 50 mM citric acid. The growth of *C. albicans* was sufficiently settled on the bottom of the wells to allow for the removal of supernatant solutions without having to centrifuge the 96-well trays. Five microliters of 5 mg/mL 5(6)-CFDA (Molecular Probes, Eugene, OR) in DMSO was

added to each well to give a final concentration of 122 μg/mL. A multichannel pipettor was used to resuspend well contents by pipetting, alternating filling and emptying the wells 20 times. The trays were then incubated in the dark at 35°C for 1 hr. The well contents were resuspended, as before, and the trays were assayed for fluorescence with a FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). 5(6)-CFDA was evaluated using excitation and emission wavelengths of 485 nm and 530 nm, respectively. The fluorescence of the drug-free control was defined as 100% and the fluorescence of the fluconazole-exposed wells were reported proportionally to this value (Fig. 2.2). The MIC endpoint was defined as the lowest fluconazole concentration at which the fluorescence was reduced to 80% of the drug-free growth control. The CFDA-microdilution method was performed in triplicate for each yeast strain assayed.

The technical time required to include the CFDA modification to the standard microdilution susceptibility test includes the 1 hr incubation time and the time required to pipette the CFDA and resuspend the yeasts in the wells.

Analysis of data. The data in Table 2.1 were determined by calculating the percentage of MICs between the two susceptibility testing methods that were identical (eg. 63.4% of MICs were identical at

24 h between the two methods). Also these data includes the percentage of the total number of MICs determined using the CFDA method that differed by a specified number of dilutions in comparison to the reference M27-A method (eg. 31.7% of MICs determined using the CFDA method were 1 dilution lower than the MICs determine using the reference M27-A method).

III. RESULTS

The comparative evaluation of the reference NCCLS broth microdilution method with the CFDA-modified microdilution method for susceptibility to fluconazole was conducted with 68 *Candida* strains, including: 53 *C. albicans*, 5 *C. tropicalis*, 5 *C. glabrata* and 5 *C. parapsilosis*. The *C. albicans* isolates chosen covered a broad range of susceptibility to fluconazole (Fig. 2.1). Some of these isolates characterisitically formed a compact "button-shaped" colony in the centre of the well and other isolates grew more dispersed across the entire bottom of the well. The MICs of control strains were within accepted limits, providing an internal control for the NCCLS reference method. Evaluation by the reference microdilution method determined that of the *Candida* strains tested, 12 strains of *C. albicans* produced

extreme trailing endpoints producing discordant MICs at 24 h (<8 μg/mL) and 48 h (≥ 64 μg/mL). They were thus considered to have the low-high MIC phenotype (20). In contrast, all *C. albicans* strains with the low-high phenotype as tested by the reference microdilution were not discordant between 24 h and 48 h by the CFDA-modified microdilution method. These *C. albicans* strains were all shown to be susceptible at both 24 h and 48 h (MIC ≤8 μg/mL). In fact, the MICs were identical for 11 of the 12 strains (MIC = 0.25 μg/mL).

The CFDA-modified microdilution method allows for the quantification of fluconazole inhibition and the production of dose-response curves (Fig. 2.2). MICs were determined from these dose-response curves after plotting the 80% inhibition and rounding up to the standardized M27-A endpoint.

Table 2.1 summarizes the distributions of the differences in MICs and the percent agreement using the reference and CFDA-modified broth microdilution methods for non-low-high phenotype strains of *C. albicans.* Considering the non-low-high phenotype *C. albicans* strains only, the CFDA-modified broth microdilution method yielded 97.6% (40 of 41 strains) agreement within ±1 dilution compared with the NCCLS reference method. At 48 h the two methods yielded 92.7% (38 of 41 strains) agreement within ±1 dilution and 97.6% (40 of 41) agreement

within ±2 dilutions. Where MIC endpoints differed between the two methods the interpretive susceptibility category changed for only one strain. The most common discrepancies between the susceptibilities of *C. albicans* to fluconazole were due to the CFDA-modified microdilution MICs being one dilution lower then the standard method. The 5 strains for each of *C. tropicalis*, *C, glabrata,* and *C. parapsilosis* showed 100% agreement within ±2 dilutions for the two methods being evaluated.

IV. DISCUSSION

Endpoint determination for fluconazole susceptibility testing is recognized to be problematic and a significant source of interlaboratory variability (6, 18, 23). The trailing growth phenomenon, which describes the partial inhibition of fungal growth over most or all of the concentration range (6, 13, 14, 23), is largely responsible for the difficulties attributed to endpoint determination, especially with *Candida* spp. (14). This so-called "trailing endpoint", which impedes azole susceptibility testing, can be exacerbated over time with some isolates, producing discordant MICs of < 8 μg/mL at 24 h and ≥ 64 μg/mL at 48 h (1, 11, 20, 21). Using the NCCLS M27-A guidelines, such discordant

MICs place these isolates into susceptible and resistant MIC interpretive categories at 24 and 48 h, respectively (12) and are conveniently referred to as having a low-high MIC phenotype (20). The current evidence suggests that the lower MIC obtained at 24 h using the reference microdilution method correlates most closely with the *in vivo* outcome (11, 20, 21).

The M27-A method establishes the endpoint for susceptibility testing of *Candida* spp. to azoles at 48 h with a criterion of 80% reduction in growth (12). Modifications of the M27-A reference method are acceptable and expected (6, 21), and it has been suggested that trailing MIC endpoints may be corrected by shortening the incubation time to 24 h and lowering the MIC endpoint to the lowest drug concentration producing a 50% reduction in growth (15, 21, 22). The requirement of a 48 h incubation for optimal testing conditions may represent a barrier to this change (7, 21). In one study (17) the results for three microdilution susceptibility test formats were shown to be reproducible and in agreement with one another after 24 h, but require 48 h to achieve acceptable agreement with the macrodilution reference MICs.

Additional efforts have proposed using a colorimetric endpoint in a microdilution format by including an oxidation-reduction indicator with the yeast and drug prior to incubation (15). However, the colorimetric

method also presents trailing endpoints at 48 h with fluconazole (15, 28) and some species-specific discrepancies have been observed (15).

The CFDA-modified microdilution format does not alter the M27-A microdilution protocol, but instead can be employed at 24 or 48 h to clarify MIC endpoints. The fluorescent dye CFDA is applied post incubation to the microdilution tray and thus does not interfere with the complex interaction between the yeast and antifungal drug.

The fluorescent dye CFDA is a lipophilic, nonpolar substrate that diffuses across the cell membrane and is hydrolyzed by nonspecific intracellular esterases to the fluorescent anion carboxyfluorescein (19). Cells with compromised membranes rapidly leak carboxyfluorescein, even when residual esterase activity is retained intracellularly (8). The utility of CFDA for assessing the vitality of *C. albicans* exposed to amphotericin B has been previously demonstrated (10), but amphotericin B was not included in this MIC study.

The CFDA-modified microdilution method allowed for the stringent 80% growth inhibition endpoint to be maintained with fluconazole susceptibility testing. Furthermore, this method permitted the evaluation of MICs at 24 or 48 h with clearly demarcated endpoints despite the trailing growth phenomenon. The CFDA-modified microdilution method determined that all 12 low-high phenotype strains of *C. albicans* were

susceptible to fluconazole and have identical endpoints at 24 and 48 h. This result supports the in vivo evidence that suggests that the strains of *C. albicans* with the low-high phenotype are actually susceptible to fluconazole (1, 20, 21). In fact, one of the low-high phenotype strains, 707-15, demonstrated to be susceptible with the CFDA-modified microdilution method has been previously shown to be susceptible *in vivo* (21).

There was excellent agreement between the NCCLS M27-A broth microdilution method and the CFDA-modified microdilution method using an 80% inhibition of growth endpoint for strains of *C. albicans* without the low-high phenotype. These results demonstrate that the CFDA-modified microdilution method for testing fluconazole is entirely comparable to the NCCLS reference microdilution method. The one strain of *C. albicans* whose MIC disagreed between the two methods was shown to be very resistant using the M27-A microdilution method and susceptible using the CFDA-modified method. This strain has unusual fluorescent staining properties and investigations are underway to determine the nature of its resistance mechanism.

A small survey of the 3 other major bloodstream fungal pathogens (16), *C. tropicalis, C. glabrata,* and *C. parapsilosis*, showed excellent agreement between the M27-A microdilution and CFDA-modified

microdilution methods (data not shown). Further studies to evaluate the applicability of the CFDA-modified method with other antifungal agents and other species of yeast are on going.

In summary, the CFDA-modified microdilution method provides objective and quantifiable endpoints for fluconazole susceptibility testing at 24 and 48 h, which are reproducible and easy to interpret. It eliminates the ambiguity of the low-high phenotype while maintaining the integrity of the NCCLS protocol. This method is simple to perform and provides the opportunity for automation and widespread use.

V. TABLES

Incubation	Percentage discrepancies between methods[b]							% Agreement	
time	≤ -2	-2	-1	0	1	2	> +2	1 dilution	2 dilutions
24 h	2.4[c]	0	31.7	63.4	2.4	0	0	97.6	97.6
48 h	2.4	4.9	34.1	51.2	7.3	0	0	92.7	97.6

[a] Only includes strains of *C. albicans* which do not have the low-high phenotype

[b] The numbers indicate the fold dilution difference (log_2)

[c] 1 of 43 strains possessed a MIC more than 2 log_2 lower by the CFDA-modified method than by the M27-A reference method

VII. FIGURES

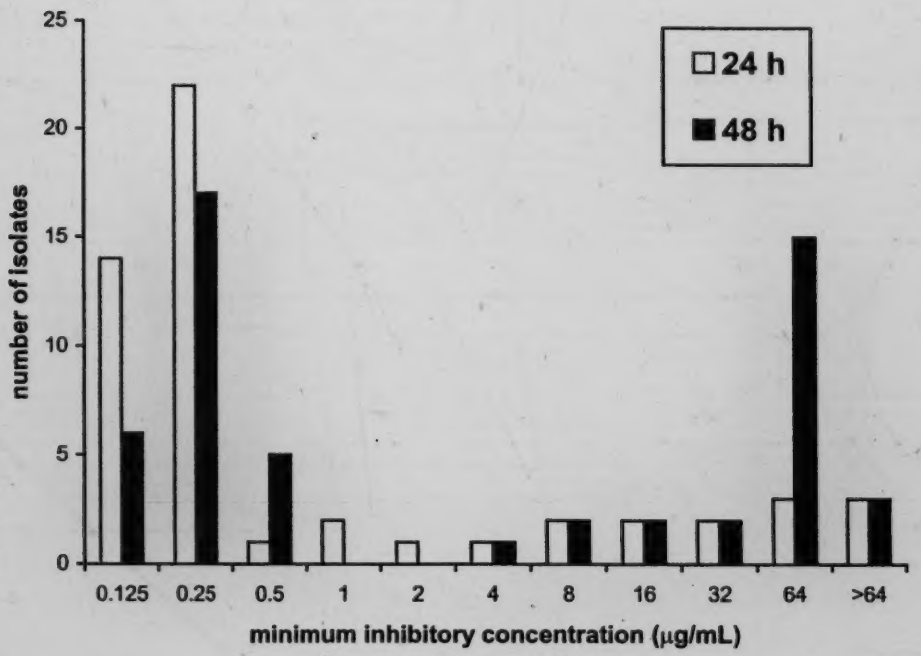
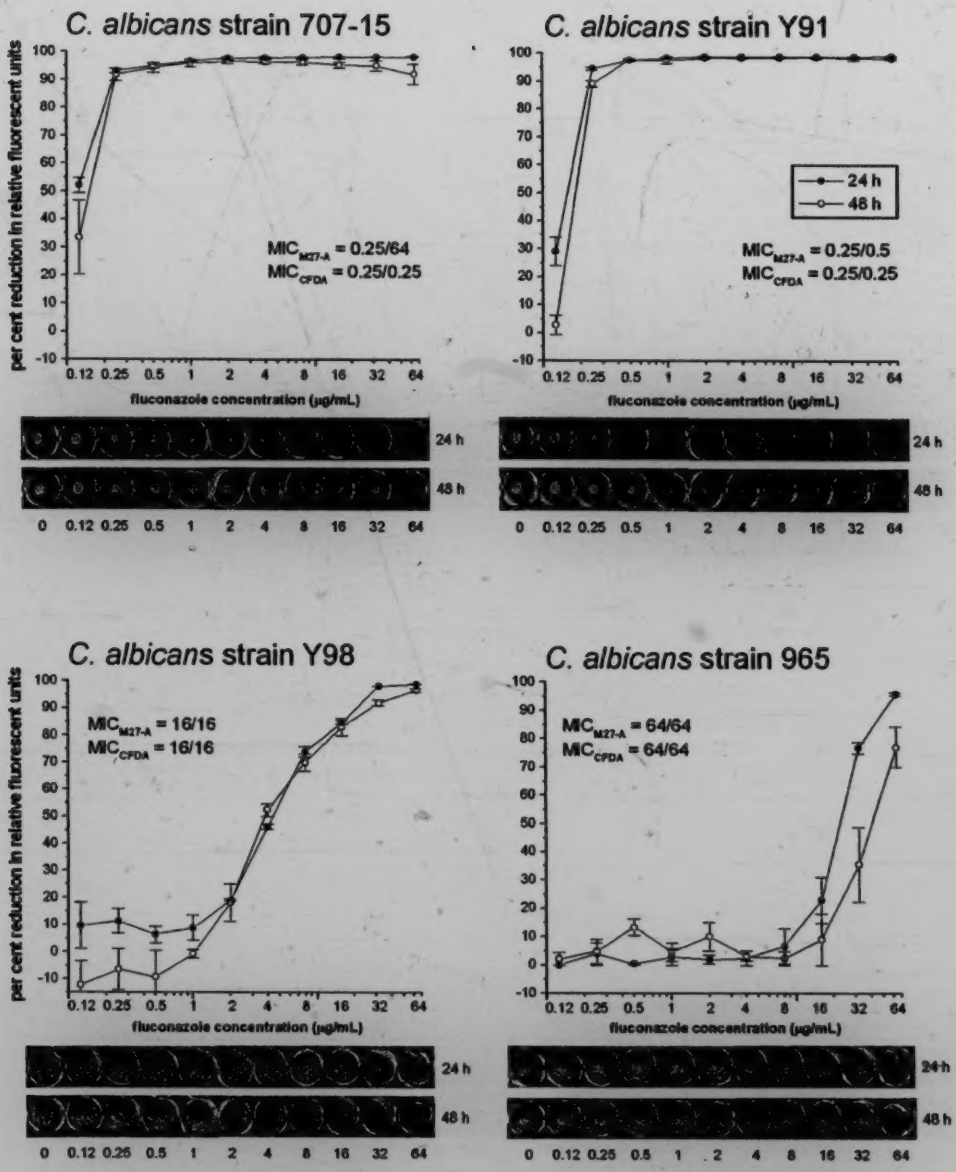


FIG. 2.1. Range of MICs to fluconazole for 53 isolates of *C. albicans* at 24 and 48 h determined with the reference M27-A broth microdilution method. The breakpoints (μg/mL) for *Candida* species against fluconazole are susceptible ≤8 μg/mL, susceptible-dose dependent 16-32 μg/mL and resistant ≥64 μg/mL [12].

FIG. 2.2. Effect of fluconazole on representative strains of *C. albicans*, which are low-high phenotype (707-15), susceptible (Y91), susceptible dose-dependent (Y98), and resistant (965). Each isolate was tested for susceptibility with the M27-A microdilution method (MIC_{M27-A}) and the CFDA-modified microdilution method (MIC_{CFDA}) at both 24 and 48 h (24/48). Results for the CFDA-modified method are shown graphically as relative fluorescent units. The fluorescence of the growth in the drug-free control was defined as 100% and the fluconazole-exposed wells were scaled to this value. Error bars indicate standard error. The results of the reference M27-A method are shown below as a digital image of the unagitated growth in the 96-well plate at 24 and 48 h.


C. albicans strain 707-15
per cent reduction in relative fluorescent units
fluconazole concentration (μg/mL)
MIC M27-A = 0.25/64
MIC CFDA = 0.25/0.25
24 h
48 h
C. albicans strain Y91
MIC M27-A = 0.25/0.5
MIC CFDA = 0.25/0.25
C. albicans strain Y98
MIC M27-A = 16/16
MIC CFDA = 16/16
C. albicans strain 965
MIC M27-A = 64/64
MIC CFDA = 64/64

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Chapter 3

Comparative Evaluation of a New Fluorescent CFDA-modified Microdilution Method for Antifungal Susceptibility Testing of *Candida albicans*

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I. INTRODUCTION

Reliable antifungal susceptibility testing of pathogenic and opportunistic fungi is of growing importance due to the emergence of antifungal-drug resistance (26), the development of new antifungal drugs (3, 12), and the increased prevalence of serious fungal infections in critically ill and immunocompromised patients (17). In 1997 the NCCLS published reference guidelines for susceptible testing of *Candida* and *Cryptococcus neoformans* against fluconazole, ketoconazole, itraconazole, flucytosine, and amphotericin B (11). The broth antifungal susceptibility testing guidelines have provided greater interlaboratory reproducibility and served as a reference for developing more efficient modifications and alternative approaches. Still, the determination of MIC endpoints remains a major source of interlaboratory variability (5, 19, 24). One of the main sources of subjective and variable endpoint interpretations is the trailing growth phenomenon caused by the partial inhibition of fungal growth over the range of antifungal concentrations (5, 8, 14, 15, 24). This is especially true for fungistatic drugs. Several studies used the colorimetric (4, 9, 16, 25) and spectrophotometric (2, 5, 13, 18) determination of broth microdilution endpoints to address this problem. Previous work has also

demonstrated the utility of using the fluorescent dye carboxyfluorescein diacetate (CFDA) in the standardized NCCLS M27-A broth microdilution method to successfully assess susceptibility of *Candida* to fluconazole (11). In this study we evaluated the applicability of the CFDA-modified microdilution method for susceptibility testing of clinical *C. albicans* isolates to flucytosine, amphotericin B, itraconazole, ketoconazole, fluconazole and voriconazole. We simultaneously compared the MIC endpoints determined using the CFDA-modified microdilution method with the microdilution MICs obtained using Alamar Blue endpoints, spectrophotometric endpoints, and the standard visual endpoints.

II. MATERIALS AND METHODS

Antifungal susceptibility testing. The reference NCCLS broth microdilution method was performed as described in the M27-A document (11). Antifungal agents (Appendix I) were diluted in RPMI 1640 medium with L-glutamine, and morpholinepropanesulfonic acid (MOPS) buffer at 165 mM and pH 7 (Angus Buffers & Biochemicals, Niagara Falls, NY) and 100 μL aliquots were placed into the wells of 96-well microtiter Linbro plates (Flow Laboratories Inc., McLean, VA) with

clear, U-shaped well bottoms. The final concentration ranges used were 0.125 to 64 μg/ml for flucytosine and fluconazole and 0.03 to 64 μg/ml for itraconazole, ketoconazole, voriconazole and amphotericin B. Six *C. albicans* strains were tested per 96-well plate, which allowed for the outermost wells to be filled with sterile water to minimize evaporation.

Five *C. albicans* colonies ≥1 mm diameter were suspended in sterile 0.85% saline and adjusted to a final concentration (after inoculation) of 0.5-2.5 x 10^3 cells per ml in RPMI 1640-MOPS medium. The inoculum was directly added to the antifungal-containing trays and incubated at 35°C without agitation. Endpoints were recorded visually, spectrophotometrically, and fluorimetrically at 24 h and 48 h. All measurements were performed in triplicate for each yeast strain assayed.

(i) Visual MIC endpoint (MIC_{visual}). The 96-well microdilution plates were first read visually without agitation as recommended by the reference NCCLS M27-A protocol (11). The reference broth microdilution method was scored by comparing the growth in each well with that in the growth control (drug-free) well. The MICs of the azole antifungals and flucytosine were defined as the minimum drug concentration at which visual growth was determined to be 80% relative

to that of the growth control (Fig. 3.1d) (11). The MIC of amphotericin B was defined as the lowest drug concentration at which there was 100% inhibition of growth compared with the growth for the drug-free control (11).

(ii) Spectrophotometric MIC endpoint (MIC_{spec}). The supernatant solutions were first removed from the microdilution plate wells using a multichannel pipettor and the remaining organisms were resuspended to 200 μl per well in 35°C-warmed 0.1 M MOPS buffer at pH 3.5 with 50 mM citric acid. The buffer resuspension step was included to allow for the same plate that was evaluated using the MIC_{visual} and MIC_{spec} methods to also be subsequently evaluated using the CFDA-modified microdilution method. A multichannel pipettor was used to mix the content of the wells 20 times. The spectrophotometric MIC was performed using a microtiter plate spectrophotometer (Diagnostics Pasteur, Chaska, MN) set at 492 nm. The absorbance of the drug-free control was defined as 100% and the absorbance of the antifungal-exposed wells were reported proportionally to this value (Fig. 3.1b). The MIC endpoints for the azoles, flucytosine, and amphotericin B were defined as the lowest concentration of antifungal drug at which the

absorbance was reduced to ≤ 50% in comparison to the drug-free growth control (2, 13, 18).

(iii) CFDA MIC endpoint (MIC_{cfda}). The CFDA-modified microdilution method (8) employs a vitality-specific fluorescent dye at 24 h and 48 h with the 96-well microdilution plates prepared according to the NCCLS M27-A protocol (11). After the removal of supernates and resuspension of yeast cells in 0.1 M MOPS buffer at pH 3.5 with 50 mM citric acid, 5 μL of 5 mg/mL 5(6)-CFDA (Molecular Probes, Eugene, OR) in DMSO was added to each well for a final concentration of 122 μg/mL. A multichannel pipettor was used to mix the well contents (20 times). The trays were then incubated without shaking in the dark at 35°C for 1 hr. The well contents were resuspended again, as before, and the trays were assayed for relative fluorescence intensity with a FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). 5(6)-CFDA was evaluated using excitation and emission wavelengths of 485 nm and 530 nm, respectively. The fluorescence of the drug-free control was defined as 100% and the fluorescence of the antifungal-exposed wells were reported proportionally to this value. The MIC endpoints were defined as the lowest concentration of antifungal drug at which the fluorescence was reduced to 80% of that of the drug-free growth control

(Fig. 3.1a, Fig. 3.2). This endpoint criteria was used for all antifungal drugs tested.

(iv) Alamar Blue MIC endpoint (MIC_{alamar}). The Alamar Blue method was identical to the reference method in terms of inoculum, drug dilution, and incubation, with the exception of the addition of the oxidation-reduction indicator Alamar Blue. Prior to the inoculation of each plate, 5 μL of 10x Alamar Blue (Trek Diagnostic Systems, Inc., Westlake, OH) was added to each well. The colorimetric MIC endpoints were read successively at 24 h and 48 h. The growth in each well was visually indicated by a color change from blue to red. Fungal isolates which exhibit trailing growth will also produce hues of purple color in transition from blue to red. To accommodate for the trailing color transition and to provide an objective and quantifiable endpoint, Alamar Blue plates were assayed for fluorescence with a FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). Alamar Blue was evaluated using the optimal excitation and emission wavelengths of 530 nm and 590 nm, respectively. The fluorescence of the drug-free control was defined as 100% and the fluorescence of the antifungal-exposed wells were reported proportionally to this value. The MIC endpoints for the azoles, flucytosine, and amphotericin B were

defined as the lowest concentrations at which the absorbance was reduced to ≤ 50% of that of the drug-free growth control (Fig. 3.1c).

III. RESULTS

A comparative evaluation of the MICs determined using 4 different broth microdilution methods for 6 antifungal drugs was conducted with 16 *C. albicans* strains (14 clinical isolates and 2 reference strains). The four methods included endpoint determinations by the standard visual estimation (MIC_{visual}), a spectrophotometric measurement (MIC_{spec}), and fluorescent measurements after incubation with either CFDA (MIC_{cfda}) or Alamar Blue (MIC_{alamar}). The 16 *C. albicans* isolates had a broad range of MICs for all of the antifungal agents assayed, as determined using the reference method with visual endpoint determination. The range of MICs for each respective agent were: 5FC (0.12 to 64 μg/mL), AMB (0.25 to 1 μg/mL), KTC (0.03 to 16 μg/mL), VRC (0.03 to 16 μg/mL), ITC (0.06 to >16 μg/mL), and FLC (0.12 to >64 μg/mL). The MIC_{50} and MIC_{90} are special variables that represent the concentrations of antifungal agent required to inhibit 50% and 90% of the *C. albicans* strains. These variables are commonly used nomenclature but it is still

helpful to reemphasize that they do not represent percent inhibition of growth. The MIC_{50}/MIC_{90} for each antifungal agent were 0.25/>64 μg/mL for 5FC, 0.5/1 μg/mL for AMB, 0.12/16 μg/mL for KTC, 0.12/>16 μg/mL for VRC, 0.25/>16 μg/mL for ITC, and 1/64 μg/mL for FLC. The MICs of two control strains, ATCC 90028 and ATCC 24433, were within the accepted limits (Table 3.1).

The CFDA-modified microdilution method allowed for the simple quantification of antifungal inhibition and the production of dose-response curves (Fig. 3.1a, Fig. 3.2). The 80% inhibition MICs were easily visualized for all the antifungals tested using these dose-response curves. Table 3.2 summarizes the overall distribution of difference and the per cent agreement in the MICs to all 6 antifungals by comparing the four microdilution methods. The MIC_{spec} and MIC_{cfda} methods both yielded greater than 80% agreement within ±1 dilution compared to the reference MIC_{visual} method at 24 h; and greater than 80% agreement within ±2 dilutions at 48 h. The MIC_{spec} and MIC_{cfda} endpoints were both lower than the MIC_{visual} endpoints at 24 h and 48 h. The highest percentage agreement of endpoints between the MIC_{visual} method compared to the MIC_{spec} and MIC_{cfda} methods, were both at 24 h within ±2 dilutions, with 97.9% and 94.7 %, respectively. Further comparison between the MIC methods using the individual antifungal drugs (Table

3.3) showed that the MIC_{cfda} endpoints for 5FC and AMB had >90% agreement within ±1 dilution compared to the MIC_{visual} endpoints at both 24 h and 48 h. The results were similar for the MIC_{spec} method, comparing the MIC_{spec} endpoints for 5FC and AMB to the MIC_{visual} endpoints, with the exception that the percentage agreement within ±1 dilution was lower; and noticeably so with AMB at 24 h. The greatest discrepancies between the MIC_{spec} and MIC_{cfda} endpoints compared to the MIC_{visual} endpoints were due to the azole antifungals at 48 h (Table 3.3). These discrepancies at 48 h were not further evaluated to see if they persisted.

Between all of the MIC methods, the highest percentage agreement was between the MIC_{cfda} and MIC_{spec} methods, yielding 92.8% agreement within ±1 dilution and 99% agreement within ±2 dilutions at 24 h; and 87.6% agreement within ±1 dilution and 98% agreement within ±2 dilutions at 48 h (Table 3.2). The greatest discrepancy between the MIC_{cfda} and MIC_{spec} methods (Table 3.2 and Table 3.3) was due to the MIC_{cfda} endpoints for FLC at 48 h being lower than the endpoints obtained using the MIC_{spec} method. Additional comparison between the MIC_{spec} and MIC_{cfda} methods for the drugs with established interpretive breakpoints (FLC, ITC, 5FC) (11), showed that all of the endpoint differences were by definition minor errors.

There was very low overall agreement between the endpoints obtained using the MIC_{alamar} method compared to those endpoints obtained using the MIC_{visual}, MIC_{spec}, and MIC_{cfda} methods (Table 3.2). This discrepancy was due to the consistently higher values obtained using the MIC_{alamar} method. The MIC_{alamar} endpoints for 5FC and AMB had >90% agreement within ±2 dilutions compared to the MIC_{visual} endpoints at both 24 h and 48 h (Table 3.3). The MIC_{alamar} endpoints for all of the azole antifungals had very low agreement in comparison to the MIC_{visual} endpoints. The best agreement that could be obtained using the MIC_{alamar} method in comparison to the MIC_{visual}, MIC_{spec}, and MIC_{cfda} methods was obtained at 24 h (Table 3.2).

Two of the *C. albicans* strains MY894 and 707-15 (Fig. 3.1, Table 3.1) tested by the reference NCCLS microdilution method using visual endpoint determinations produced extreme trailing endpoints with ketoconazole, voriconazole, itraconazole, and fluconazole. These strains which manifested partial inhibition of growth over the entire range of azoles resulted in discordant MICs at 24 h (sensitive) and 48 h (resistant). These strains when evaluated using both MIC_{cfda} and MIC_{spec} methods were not discordant at 24 h versus 48 h, but were susceptible to all of the azoles at 24 h and 48 h. The MICs for the low-high phenotype strain MY894 are shown in figure 3.1. The MICs using

the MIC_{cfda} method (80% inhibition) and the MIC_{spec} method (50% inhibition) were ≤0.12 μg/mL at both 24 h and 48 h. However, the MIC obtained using the MIC_{alamar} method (50% inhibition) was 4.0 μg/mL at 24 h and >64 μg/mL at 48 h. The MIC obtained using the reference MIC_{visual} method (80% inhibition) was 0.5 μg/mL at 24 h and trailed to >64 μg/mL at 48 h.

IV. DISCUSSION

We have previously demonstrated the utility of CFDA for susceptibility testing of fluconazole with *Candida albicans* (8). Using of CFDA for assessing the vitality of *C. albicans* exposed to amphotericin B under different conditions has also been previously demonstrated (7). We report here for the first time, the use of a CFDA-modified broth microdilution method for antifungal susceptibility testing of *C. albicans*. The CFDA-modified microdilution format does not alter the M27-A microdilution protocol (11). The fluorescent dye CFDA is added to the microdilution tray at the 24 h or 48 h endpoint and does not interfere in any way with the interaction between the fungal cells and antifungal agent. In this manner, the different endpoints can be sequentially

determined: visually (MIC_{visual}), spectrophotometrically (MIC_{spec}) and fluorescently (MIC_{cfda}), all with the same 96-well microdilution plate. The subjective nature of visual endpoint determination and the difficulty with partial inhibition of fungal growth that occurs with azole antifungals was responsible for the higher endpoints derived using the MIC_{visual} method, especially at 48 h (Table 3.3). Interestingly, compared to the MIC_{spec} and MIC_{cfda} methods, the MIC_{spec} endpoints had lower agreement to the MIC_{visual} endpoints for amphotericin B and flucytosine, especially amphotericin B at 24 h. Due to the relative ease in determining visual endpoints for flucytosine and amphotericin B, this result suggests an advantage of the MIC_{cfda} method over the MIC_{spec} method.

The spectrophotometric measurement of turbidity to evaluate the inhibition of fungal growth (MIC_{spec}) has been previously shown to provide an objective measurement and correlate well with the reference method (2, 5, 13, 18). The MIC_{cfda} microdilution method is a completely different methodological way of evaluating viability. CFDA is a fluorescent, nonpolar substrate that diffuses across the cell membrane and is hydrolyzed by nonspecific intracellular esterases to the fluorescent anion carboxyfluorescein (20). The MIC_{cfda} and MIC_{spec} endpoints are also differentially measured at 80% and 50% inhibition compared to the growth control, respectively. Thus, the MIC_{cfda} method

provides an objective and independent confirmation of the MIC_{spec} at both 24 h and 48 h. The greatest difference in endpoints between the MIC_{cfda} and MIC_{spec} methods was observed with fluconazole at 48 h (Table 3.3). The MIC_{spec} method may exaggerate the partial inhibition of growth by fluconazole in some strains producing trailing endpoints.

Colorimetric endpoint determination of microdilution susceptibility testing was previously demonstrated to improve endpoint reading under some circumstances (4, 9, 16, 25). However, the colorimetric microdilution method has also been shown to be difficult to evaluate at 48 h due to the partial inhibition of growth with azole antifungals (16, 25), and some species-specific discrepancies have been observed (9, 16). We also have shown extensive trailing of the azole endpoints with the MIC_{alamar} method at 48 h (Table 3.2). There was good agreement between the MIC_{alamar} and MIC_{visual} methods for amphotericin B and flucytosine endpoints (Table 3.3). However, there was also less than 50% agreement ±1 dilution at 24 h for azole endpoints using the MIC_{alamar} method compared to each of the three other MIC methods.

The trailing growth phenomenon, which describes the partial inhibition of fungal growth over most or all of the concentration range for azole antifungals (5, 14, 15, 24), is responsible for most of the difficulties encountered with endpoint determination. The resulting "trailing

endpoint" is exacerbated over time with some strains, producing discordant MICs (MIC_{visual}) of < 8 μg/mL at 24 h and of ≥ 64 μg/mL at 48 h (1, 8, 21, 22). Such discordant MICs place these isolates into susceptible and resistant MIC interpretive categories at 24 h and 48 h, respectively (11), and are referred to as having a low-high MIC phenotype (8, 21). In this study, we included 2 strains (MY894 and 707-15) with such discordant MICs (MIC_{visual}) to fluconazole, ketoconazole, itraconazole, and voriconazole. We previously showed that the CFDA-modified microdilution method could identify 12 low-high phenotype strains of *C. albicans* to be susceptible to fluconazole at both 24 h and 48 h (8). The two low-high phenotype strains examined in this study using the MIC_{cfda} method were also shown to be susceptible at both 24 h and 48 h to fluconazole, ketoconazole, itraconazole, and voriconazole. The MIC_{spec} confirmed these results. These observations are supported by current evidence that suggests that the lower MIC at 24 h using the reference method (MIC_{visual}) correlates best with the *in vivo* outcome (10, 21, 22). The MIC_{alamar} method determined both of the low-high phenotype strains to have endpoints at 48 h which were resistant to the azole antifungals.

The endpoints for susceptibility testing of *Candida* species. to flucytosine and azole antifungals were established by the M27-A

protocol with a criterion of 80% reduction in growth after 48 h of incubation (11). It has been suggested that the trailing azole MIC endpoint phenomenon may be reduced by lowering the MIC endpoint to a 50% reduction in growth and by shortening the incubation time to 24 h (16, 22, 23). The requirement of a 48 h incubation for optimal testing conditions may represent a barrier to this change (6, 18, 22). It is important that the selection of a 24 h or 48 h incubation time be based on the best agreement with clinical outcome, without overdue consideration for difficulties encountered with *in vitro* trailing endpoints to the azole antifungals. The results described here demonstrate that the trailing growth phenomenon does not effect the MIC_{cfda} or MIC_{spec} endpoint determinations at 48 h, unlike both the MIC_{alamar} and MIC_{visual} methods. Interestingly, for all of six of the antifungals tested, only 33% of all endpoints differed from 24 h to 48 h using the MIC_{cfda} method. In comparison, 46% of all the endpoints tested differed from 24 h to 48 h using the MIC_{spec} method.

In summary, there was excellent agreement between the reference broth microdilution method (MIC_{visual}) and the CFDA-modified (MIC_{cfda}) and spectrophotometric methods (MIC_{spec}). The MIC_{cfda} and MIC_{spec} methods provided objective, clearly demarcated and reproducible endpoints for antifungal susceptibility testing at 24 h and 48 h, while

maintaining the integrity of the NCCLS protocol. Consequently, an even higher percentage agreement was observed between the MIC_{cfda} and MIC_{spec} methods. Both the MIC_{cfda} and MIC_{spec} methods eliminated the ambiguity of the trailing growth phenomenon for all of the azole antifungal drugs, including the two low-high phenotype strains. These two methods used in combination could provide a powerful tool for antifungal susceptibility testing and increase interlaboratory reproducibility.

V. TABLES

TABLE 3.1. Comparison between microdilution MICs (μg/mL) for *C. albicans* clinical and reference strains after 24 h and 48 h of incubation and determined by visual (MIC_{visual}), spectrophotometric (MIC_{spec}), Alamar Blue (MIC_{alamar}), and CFDA (MIC_{cfda}) methods

ATCC 90028 (24-/48-h)[a, b]

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	1.0/2.0	0.25/1.0	0.12/1.0	0.5/1.0
AMB	0.5/1.0	0.5/1.0	0.5/1.0	0.5/1.0
KTC	0.03/0.03	0.03/0.03	0.03/0.03	0.03/8.0
VRC	0.03/0.03	0.03/0.03	0.03/0.03	0.06/>64.0
ITC	0.25/0.25	0.06/0.25	0.06/0.06	1.0/>64.0
FLC	0.25/0.5	0.25/0.5	0.12/0.12	1.0/>64.0

ATCC 24433 (24-/48-h)

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	0.5/1.0	0.5/1.0	0.5/1.0	1.0/2.0
AMB	0.5/0.5	0.5/0.5	0.5/1.0	2.0/2.0
KTC	0.03/0.03	0.03/0.03	<0.03/<0.03	0.06/8.0
VRC	0.03/0.03	0.03/0.03	<0.03/<0.03	0.12/>64.0
ITC	0.25/0.25	0.25/0.25	0.25/0.25	0.5/>64.0
FLC	0.25/0.12	0.25/0.25	<0.12/<0.12	1.0/>64.0

MY894 Low-High Phenotype (24-/48-h)

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	0.12/2.0	0.25/1.0	0.25/1.0	0.5/4.0
AMB	0.12/0.25	0.03/0.12	0.12/0.12	0.25/1.0
KTC	0.03/16.0	0.03/0.03	0.03/0.03	0.12/8.0
VRC	0.03/>16.0	0.03/0.03	0.03/0.03	0.25/>16.0
ITC	0.12/>16.0	0.12/0.25	0.12/0.12	0.25/>16.0
FLC	0.5/>64.0	0.12/0.12	0.12/0.12	4.0/>64.0

707-15 Low-High Phenotype (24-/48-h)

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	0.12/0.25	0.12/0.25	0.12/0.25	0.25/0.25
AMB	0.5/1.0	0.5/1.0	0.5/1.0	1.0/1.0
KTC	0.03/16.0	0.03/0.03	<0.03/<0.03	16.0/8.0
VRC	0.03/>16.0	0.03/0.03	<0.03/<0.03	>16.0/>16.0
ITC	0.25/>16.0	0.25/0.25	0.25/0.25	>16.0/>16.0
FLC	0.25/>64.0	0.25/0.25	<0.12/<0.12	>64.0/>64.0

MY3317 (24-/48-h)

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	0.12/0.25	0.12/0.25	0.12/0.25	0.25/4.0
AMB	0.12/0.25	0.12/0.25	0.06/0.25	0.25/1.0
KTC	0.03/0.03	0.03/0.06	0.03/0.03	4.0/8.0
VRC	0.03/0.06	0.03/0.06	0.03/0.03	>16.0/>16.0
ITC	0.03/0.06	0.03/0.06	0.03/0.03	>16.0/>16.0
FLC	0.12/0.5	0.12/0.5	0.12/0.12	>64.0/>64.0

Y86 (24-/48-h)

Drug	MIC method			
	visual	spec	cfda	alamar
5FC	0.12/0.12	0.12/0.12	0.12/0.12	0.12/0.12
AMB	0.5/1.0	0.25/0.5	0.25/0.5	0.25/0.5
KTC	0.12/0.5	0.03/0.12	0.03/0.03	1.0/8.0
VRC	0.12/0.5	0.03/0.25	0.03/0.03	8.0/>16.0
ITC	0.03/0.25	0.03/0.25	0.03/0.03	2.0/>16.0
FLC	4.0/8.0	2.0/8.0	2.0/2.0	>64.0/>64.0

[a]values separated by (/) indicate the MIC endpoints at 24 h and 48 h

[b]the microdilution MIC endpoints were defined as the lowest drug concentrations that produced reductions of 80% (visual), 50% (spectrophotometric), 80% (CFDA), and 50% (Alamar), respectively, in comparison to that of the drug-free control

TABLE 3.2. Summary of the distribution of differences between the broth microdilution MIC endpoints of 16 strains of *C. albicans* to all 6 antifungal agents at 24 h and 48 h as determined by the comparison of alternative methods (spectrophotometric, Alamar, and CFDA) with the reference visual endpoint

Incubation time(h)	% Discrepancies between MIC_{cfda} compared to $MIC_{spectrophotometric}$ [a]							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	1	3.1	19.8	66.7	6.3	3.1	0	92.8	99
48	1	7.3	16.7	63.6	7.3	3.1	1	87.6	98
Incubation time(h)	% Discrepancies between MIC_{cfda} compared to MIC_{visual}							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	5.2	8.3	20.8	62.5	2.1	1	0	85.4	94.7
48	16.7	12.5	17.7	51	2.1	0	0	70.8	83.3
Incubation time(h)	% Discrepancies between MIC_{alamar} compared to MIC_{cfda}							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	0	0	1	22.9	24	17.7	34.4	47.9	65.6
48	0	0	0	14.6	12.5	7.3	65.6	27.1	34.4
Incubation time(h)	% Discrepancies between $MIC_{spectrophotometric}$ compared to MIC_{visual}							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	1	11.5	13.5	67.7	5.2	0	1	86.4	97.9
48	11.5	9.4	17.7	57.3	3.1	1	0	78.1	88.5
Incubation time(h)	% Discrepancies between MIC_{alamar} compared to $MIC_{spectrophotometric}$							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	0	0	0	22.9	29.2	16.7	31.3	52.1	68.8
48	0	0	0	13.6	9.4	6.3	70.8	23	29.3
Incubation time(h)	% Discrepancies between MIC_{alamar} compared to MIC_{visual}							% Agreement within number of dilutions	
	<-2	-2	-1	0	1	2	>2	1	2
24	0	0	5.2	24	22.9	24	24	52.1	76.1
48	0	0	4.2	20	13.5	7.3	55.2	37.7	45

[a] The numbers -2 to 2 indicate the fold dilution difference (log2).

TABLE 3.3. Distribution of difference between the broth microdilution MIC endpoints of 16 strains of C. albicans to each of 6 antifungal agents at 24 h and 48 h as determined by the comparison of alternative methods (spectrophotometric, Alamar, and CFDA) with the reference visual endpoint

Drug	Incubation time(h)	% Discrepancies between MIC_{cfda} compared to $MIC_{spectrophotometric}$[a]							% Agreement within number of dilutions	
		<-2	-2	-1	0	1	2	>2	1	2
5FC	24	6.3		6.3	68.8	18.8			93.8	93.8
	48				87.5		6.3	6.3	87.5	93.8
AMB	24			12.5	62.5	12.5	12.5		87.5	100
	48				56.3	43.8			100	100
KTC	24			6.3	87.5	6.3			100	100
	48			6.3	75	18.8			100	100
VRC	24			18.8	81.3				100	100
	48	6.3		25	68.8				93.8	93.8
ITC	24		6.3	12.5	68.8	6.3	6.3		87.5	100
	48	18.8		12.5	68.8				81.3	81.3
FLC	24		6.3	56.3	37.5				93.8	100
	48		25	43.8	31.3				75	100

Drug	Incubation time(h)	% Discrepancies between MIC_{cfda} compared to MIC_{visual}							% Agreement within number of dilutions	
		<-2	-2	-1	0	1	2	>2	1	2
5FC	24	6.3		6.3	75	12.5			93.8	93.8
	48			12.5	87.5				100	100
AMB	24		6.3	43.8	50				93.8	100
	48			18.8	68.8	12.5			100	100
KTC	24		6.3	12.5	81.3				93.8	100
	48	25	12.5	18.8	43.8				62.5	75
VRC	24		6.3	25	68.8				93.8	100
	48	18.8	12.5	25	43.8				68.8	81.3
ITC	24	6.3	12.5	6.3	68.8		6.3		75	93.8
	48	31.3	12.5	18.8	37.5				56.3	68.8
FLC	24	12.5	18.8	37.5	31.3				68.8	87.5
	48	25	43.8	6.3	25				31.3	75

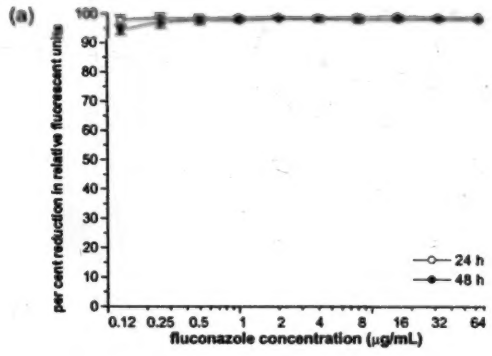
Drug	Incubation time(h)	% Discrepancies between $MIC_{spectrophotometric}$ compared to MIC_{visual}							% Agreement within number of dilutions	
		<-2	-2	-1	0	1	2	>2	1	2
5FC	24	6.3	6.3	18.8	62.5	6.3			87.5	93.8
	48	6.3	6.3	12.5	75				87.5	93.8
AMB	24	6.3	18.8	31.3	43.8				75	93.8
	48		6.3	43.8	50				93.8	100
KTC	24		6.3		87.5	6.3			93.8	100
	48	18.8	12.5	12.5	43.8	6.3	6.3		62.5	81.3
VRC	24		6.3	6.3	87.5				93.8	100
	48	12.5	12.5	6.3	68.8				75	87.5
ITC	24		12.5	6.3	81.3				87.5	100
	48	12.5	12.5	12.5	62.5				75	87.5
FLC	24		18.8	25	37.5	18.8			81.3	100
	48	18.8	12.5	18.8	37.5	12.5			68.8	81.3

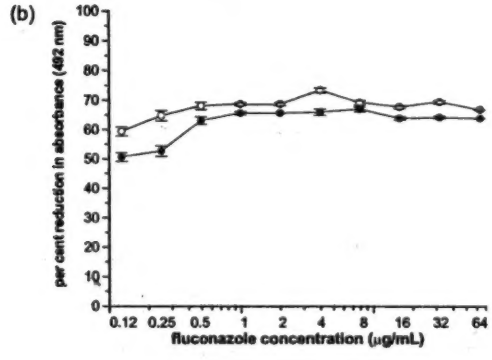
Drug	Incubation time(h)	% Discrepancies between MIC_{alamar} compared to MIC_{visual}							% Agreement within number of dilutions	
		<-2	-2	-1	0	1	2	>2	1	2
5FC	24			6.3	43.8	31.3	12.5	6.3	81.3	93.8
	48			6.3	50	37.5		6.3	93.8	93.8
AMB	24			18.8	31.3	37.5	12.5		87.5	100
	48			6.3	18.8	37.5	37.5		62.5	100
KTC	24				31.3	12.5	25	31.3	43.8	68.8
	48			12.5	6.3			81.3	18.8	18.8
VRC	24				31.3	6.3	18.8	43.8	37.5	56.3
	48				12.5			87.5	12.5	12.5
ITC	24				6.3	25	31.3	37.5	31.3	62.5
	48				12.5			87.5	12.5	12.5
FLC	24			6.3	6.3	31.3	31.3	25	43.8	75
	48				18.8	6.3	6.3	68.8	25	31.3

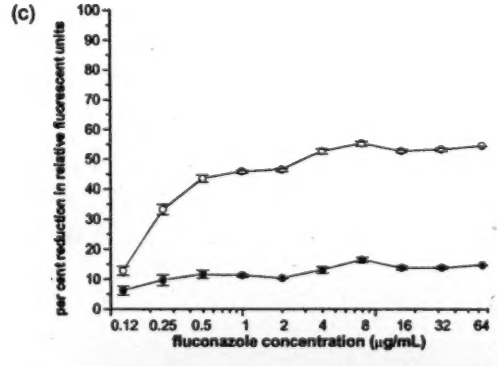
[a] The numbers –2 to 2 indicate the fold dilution difference (log_2)

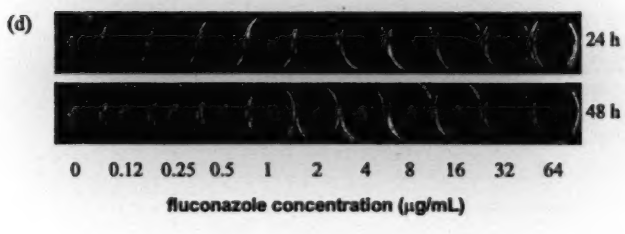
VI. FIGURES

FIG 3.1. Fluconazole MIC endpoints at 24 h and 48 h for the low-high phenotype *C. albicans* strain MY894 using four microdilution MIC methods: (a) the CFDA-modified method (MIC_{cfda}), endpoint is 80% inhibition of control fluorescence, (b) the spectrophotometric method (MIC_{spec}), endpoint is 50% inhibition of control fluorescence, (c) the Alamar Blue method (MIC_{alamar}), endpoint is 50% inhibition of control fluorescence, and (d) the visual endpoint determination of the reference NCCLS microdilution method (MIC_{visual}), endpoint is 80% inhibition of control growth. The results for the MIC_{cfda}, MIC_{spec}, and MIC_{alamar} methods are shown graphically. The fluorescence or optical density of the growth in the drug-free control was defined as 100% and the fluconazole-exposed wells were scaled to this value. The results of the MIC_{visual} reference M27-A method are shown as a digital image of the unagitated growth in the 96-well plate at 24 h and 48 h.


(a)
per cent reduction in relative fluorescent units
24 h
48 h
0.12 0.25 0.5 1 2 4 8 16 32 64
fluconazole concentration (μg/mL)


(b)
per cent reduction in absorbance (492 nm)
0.12 0.25 0.5 1 2 4 8 16 32 64
fluconazole concentration (μg/mL)


(c)
per cent reduction in relative fluorescent units
0.12 0.25 0.5 1 2 4 8 16 32 64
fluconazole concentration (μg/mL)


(d)
24 h
48 h
0 0.12 0.25 0.5 1 2 4 8 16 32 64
fluconazole concentration (μg/mL)

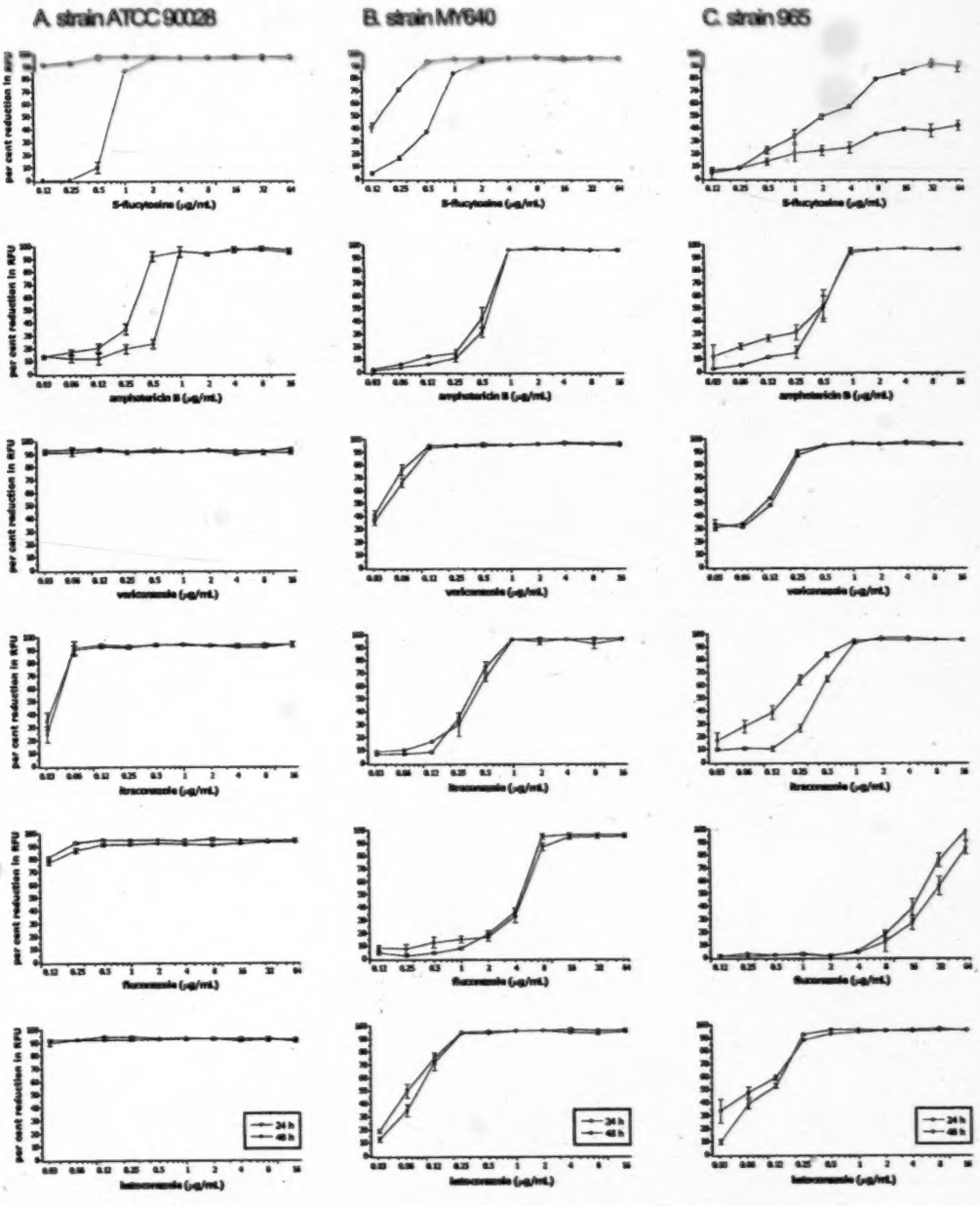


FIG. 3.2. The CFDA-modified microdilution method for 5-flucytosine, amphotericin B, ketoconazole, voriconazole, itraconazole, and fluconazole with 3 representative strains of *C. albicans*: (a) ATCC 90028, (b) MY640, and (c) 965 at 24 h and 48 h. The fluorescence of the growth in the drug-free control is defined as 100%. The error bars represent standard error.

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Chapter 4

The Measurement of Intracellular ATP for the Rapid Susceptibility Testing of *Candida albicans* to Amphotericin B and Fluconazole

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I. INTRODUCTION

The clinical interpretation of antifungal susceptibility testing remains somewhat controversial (13). Uncertainty remains over the optimal reading of MIC end points and the duration of incubation for antifungal susceptibility testing (10).

The partial inhibition or trailing effect that is commonly observed with the azole drugs precludes the determination of well-defined endpoints and creates a great deal of variability and confusion (7). Establishing a clear correlation between *in vitro* susceptibility testing using amphotericin B and patient outcome has also been difficult (28). Amphotericin B remains the drug of choice for systemic fungal infections (20, 28) and consequently a more rapid susceptibility test would be helpful (8, 9).

There is a positive correlation between fungal ATP concentrations and the number of viable cells within a given population (29), because all metabolic pathways are stoichiometrically related through the adenine nucleotide system, (4) and ATP is rapidly destroyed after cellular death (12). A sensitive and reproducible assay was developed and optimized for measuring intracellular ATP in fungi. The assay was originally developed for the assessment of vitality in bacteria and fungi. The

purpose of this study was to evaluate the feasibility and benefits of antifungal susceptibility testing of *C. albicans* for amphotericin B and fluconazole using measurements of intracellular ATP.

II. MATERIALS AND METHODS

Macrodilution susceptibility testing ($MIC_{reference}$). The reference NCCLS broth macrodilution method was performed as described in the M27-A document (21). Antifungal stock solutions were diluted in RPMI 1640 medium with L-glutamine, and morpholinepropanesulfonic acid (MOPS) buffer at 165 mM and pH 7 (Angus Buffers & Biochemicals, Niagara Falls, NY) and serial twofold dilutions were prepared in 12 x 75 mm tubes (Appendix I). The final concentration range used was 0.03 to 16 μg/mL for amphotericin B and 0.125 to 64 μg/mL for fluconazole. Five colonies of *C. albicans* of ≥1 mm diameter were suspended in sterile 0.85% saline. The cell density was adjusted to a 0.5 McFarland standard and diluted in RPMI-MOPS medium to a final concentration of 0.5-2.5 x 10^3 cells per mL. Each tube received 0.1 mL of antifungal drug and 0.9 mL of adjusted inoculum. The tubes were incubated at 35°C without agitation. Visual endpoints were recorded after 48 h of

incubation. The $MIC_{reference}$ method was scored by comparing the growth in each tube with that in the growth control (drug-free) tube.

The MIC of amphotericin B was defined as the minimum drug concentration at which there was 100% inhibition of growth compared with the growth for the drug-free control (21). The MIC of fluconazole was defined as the minimum drug concentration at which visual growth was determined to be 80% relative to that of the growth control (21).

5,(6)-carboxyfluorescein diacetate (CFDA)-modified macrodilution susceptibility test (MIC_{CFDA}). The 1 mL culture tubes were centrifuged at 9,300 × *g* and the pellet was resuspended in MOPS buffer (0.1 M MOPS, pH 7), washed 2 more times and resuspended a final time in MOPS buffer (pH 3) plus 50 mM Citric Acid. Ten microliters of 5 mg/mL CFDA (Molecular Probes, Eugene, OR) in DMSO was added to each 1 mL sample for a final concentration of 50 μg/mL. The culture was incubated with the stain in the dark at 35°C with shaking for 45 min. Stained culture samples were distributed (200 μL per well) in triplicate into a 96-well Nunc-Immuno PolySorp plate (Nunc, Nalge Nunc International, Rochester, NY) and assayed for relative fluorescence intensity using a FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). Excitation and emission wavelengths

were 485 and 530, respectively. Fluconazole susceptibility testing was conducted by the CFDA macrodilution procedure and endpoints were defined as the lowest concentration of fluconazole that produced ≥80% decrease in CFDA fluorescent intensity compared to the positive control culture (MIC_{CFDA}).

Intracellular ATP measurements of *C. albicans*

ATP luciferase assay. The concentration of ATP was assayed by measuring the luminescence produced by the oxidation of luciferin in the presence of luciferase, ATP, oxygen and magnesium ions (6). When the other components are present in excess, then the luciferase reaction is quantitatively specific for ATP (18). The amount of light generated is directly proportional to the original concentration of ATP in the reaction mixture. Luciferase has an extremely high specificity towards ATP and no other naturally occuring compound or ribonucleoside triphosphate is known to substitute for ATP (17). Important experimental variables of the ATP luciferase assay include the maintenance of the ionic composition, an optimal pH of 7.75 and an optimal temperature of 21 to 22 °C (1, 6).

Analytical equipment and reagents. Light emission from the bioluminescence assay was measured in a Bio-Orbit (Turku, Finland) 1258 microplate luminometer. The luminescence reaction temperature was set internally to 21°C. The ATP assay mix (Sigma Chemical Co.) containing luciferin and luciferase was prepared fresh according to the manufacturer's instructions. Apyrase (purified grade I; Sigma Chemical Co.) was used to eliminate extracellular ATP before extracting intracellular ATP. Reagents were prepared using sterile, distilled water and were monitored for contaminating ATP using the luciferase ATP assay. The assay was performed immediately and protected from the light.

Elimination of extracellular ATP. The 1 mL culture tubes were centrifuged at 9,300 × *g* and the pellets were resuspended in 50 µL of Tris-EDTA (TE) buffer (0.1 M Tris buffer [pH 7.8] containing 2 mM EDTA). The 50 µL washed sample was incubated for 15 min at 37°C with 50 µL of 0.04% apyrase ATPase.

Optimization of ATP extraction method. A variety of methods have been published for extracting ATP from cells and microorganisms. Different ATP extractants that have been used include: (i) boiling buffers

(22); (ii) various dilute acids such as trichloroacetic acid and perchloric acid (5); (iii) organic solvents such as dimethyl sulphoxide, acetone and ethanol (1, 11, 31); and (iv) surfactants such as Triton X-100 and benzalkonium chloride (5, 14). The conditions appropriate for extracting ATP from biological material are often unsuitable for luciferase activity. An ideal extraction protocol should produce a high yield of ATP, little inhibition of the luciferase reaction, and stable light output. The turnover of ATP is high in most biological systems and sampling and extracting ATP must be rapid to minimize metabolic changes (4).

The thick cell wall of fungal cells required a strong extractant or a long extraction time with detergents. Boiling buffer has been shown to terminate all metabolic activity immediately (4). A sample being extracted for ATP was diluted with boiling buffer to rapidly attain a sufficiently high temperature and denature the ATP-degrading enzymes. The extraction temperature was important, because a reduction in temperature of the buffered solution of a few degrees below 100 °C reduced the ATP yield. Thus, the boiling buffer method did inhibit luciferase activity but also lost some sensitivity due to the sample dilution.

Extraction of intracellular ATP. After eliminating extracellular ATP, 50 µL of the apyrase-treated sample was pipetted into 300 µL of boiling Tris-EDTA buffer. After boiling for 90 s, the extracts were cooled in an ice bath and immediately frozen at -75°C for later analysis. This procedure inactivated the apyrase ATPase and disrupted the fungal cells. Additional ATPase activity was not present when incubated with ATP.

Measurement of intracellular ATP. The ATP assay mix (100 µL) was added to 200 µL of each thawed sample extract (in triplicate) in a nonstandard 96-well opaque plate (Corning Costar Corp., Cambridge, MA). The intensity of the luminescence was determined photometrically for 10 s after 1 min of incubation at 560 nm. The ATP concentration present in the sample extracts was determined with an ATP standard curve.

Minimizing experimental error. Contaminating ATP can be easily introduced into a sample to produce false-positive results unless great care is taken (5, 32). There are also a number of situations in which the luciferase ATP assay results can be falsely low. Cell extracts can cause a decrease in the light output by inactivating the luciferase enzyme or by

containing quenching materials. Methods that employ adding an extractant often reduce the activity of the luciferase enzyme and they must be diluted before carrying out the ATP luciferase assay (30). Inhibition of the luciferase reaction was evaluated by measuring the intensity of light emitted from a buffered cell extract after a standard amount of ATP has been added in comparison to the light emitted from an equal concentration of ATP in buffer alone. Incomplete inactivation of ATP-converting enzymes in the extract may also cause the light output to decrease when using the ATP luciferase assay (17). Thus, EDTA was included in the extraction medium and to irreversibly inactivate ATP-converting enzymes.

III. RESULTS

Intracellular ATP measurements. The standard curve for the concentration of intracellular ATP versus luminescence intensity is shown in Fig. 4.2. The luminescence measured between the range of 10^4 to 10^9 RLU was directly proportional to the ATP concentration in a range from 10^{-11} and 10^{-6} M. This measurement range was similar to previous reported findings obtained with comparable experimental

conditions (2, 17). A linear relationship between the luminescence intensity and the number of viable *C. albicans* cells determined by plate count was also measured (Fig. 4.1). The minimum number of replicating and healthy *C. albicans* cells that could be detected with the described methodology was estimated to be between approximately 100 and 1000 cells.

A known concentration of ATP (5 μL of $1x10^{-5}$ M) was added to the intracellular extracts for use as a control for the inhibition of the luciferase reaction. Similar controls with ATP were used to confirm the inactivation of the apyrase ATPase and the stability of ATP in the boiling buffer extraction procedure. The highest concentration of DMSO used in the study did not have an impact on the intracellular ATP concentration or the growth of *C. albicans* on agar (data not shown). Corrections for background luminescence were made by direct subtraction.

Fluconazole. The measurement of intracellular ATP was used to evaluate 12 low-high phenotype strains, 1 susceptible strain and 1 resistant strain of *C. albicans* for susceptibility to fluconazole (Fig. 4.3, Fig. 4.4). The endpoint for fluconazole susceptibility testing using the MIC_{ATP} method was defined as the lowest concentration of antifungal

drug that caused ≥90% decrease in intracellular ATP compared to the positive control culture (MIC_{ATP}). The low-high phenotype strains were all shown to be susceptible at 12, 24 and 48 hr using the MIC_{ATP} method. The MIC_{ATP} endpoints for each individual low-high phenotype strain were identical at the three incubation times. Results obtained using the MIC_{ATP} method gave the same endpoints at 24 hr as those obtained by the $MIC_{reference}$ method. In addition, there was 93% essential agreement between the endpoints determined using the MIC_{ATP} method and CFDA-modified macrodilution method (MIC_{CFDA}) (Fig. 4.4). This agreement was calculated as the percentage of MICs determined using the MIC_{ATP} method that were the same as those obtained using the MIC_{CFDA} method. The discrepancies present were due to the MIC_{ATP} endpoints being 1 dilution lower at 12 hr. There was no change in the interpretive susceptibility category where these MIC endpoints differed (susceptible MIC is ≤8 μg/mL, susceptible dose-dependent MIC is 16-32 μg/mL and resistant MIC is ≥64 μg/mL). The shared MIC_{ATP} and MIC_{CFDA} endpoints at 12, 24 and 48 hr also coincided with an 80% reduction in plate counts at each time point. An increase in the intracellular ATP, CFDA fluorescence, and plate counts occurred between 12 and 24 hr over the entire fluconazole

concentration range. After 24 hr, fungistatic inhibition prevented the three parameters from increasing further.

Amphotericin. The comparative evaluation of the $MIC_{reference}$ method and the MIC_{ATP} method for susceptibility to amphotericin B was determined for 12 strains of *C. albicans*. The MIC_{ATP} of amphotericin B was defined as the lowest concentration of antifungal drug that had a ≥99% decrease in the intracellular ATP compared to the growth control culture. The susceptibility to amphotericin B for these strains covered a range of MICs between 0.25 and 1.0 μg/mL. The MIC_{ATP} method yielded 100% agreement when measured at 0.5, 6 and 48 hr compared to the $MIC_{reference}$ method when measured at 24 and 48 hr (Fig. 4.5). There was 75% agreement between the macrodilution and intracellular ATP methods after an incubation of 30 min when the MIC_{ATP} endpoint was defined as ≥60% inhibition compared to the nonexposed culture (Fig. 4.5).

IV. DISCUSSION

The concentration of intracellular ATP has been shown to be directly proportional to the plate count viability for *C. albicans* treated with several azole antifungal agents (2). Two previous studies have correlated fluconazole MICs to the total concentration of ATP present in the culture (14, 34). In these studies ATP measurements by Kretschmar *et al.* (14) and Yoshida *et al.* (34) used different assays with proprietary chemical extractants that measured the sum of both intracellular and extracellular ATP (14, 34). In both studies, the fluconazole MICs were shown to correlate best when the MIC endpoint, determined by measuring total ATP, was defined as the lowest drug concentration at which there was a 50% inhibition of luminescence compared with the growth for the drug-free control.

We used the measurements of intracellular ATP in combination with the NCCLS macrodilution method (MIC_{ATP}) to first evaluate the susceptibility of 12 low-high phenotype strains of *C. albicans* to fluconazole. These strains had discordant MICs of <8 μg/mL at 24 hr and of ≥64 μg/mL at 48 hr using the NCCLS microdilution and macrodilution broth susceptibility tests. Despite the extreme trailing growth that occurred with these strains, a much higher endpoint, 90%

inhibition, could be applied when measuring only intracellular ATP at 12, 24 and 48 hr (Fig. 4.4B). We previously showed the 12 low-high phenotype strains to be susceptible to fluconazole at 24 and 48 hr with a CFDA-modified microdilution method (16). These results were confirmed using the MIC_{ATP} method. There was complete agreement between the MIC_{ATP} and MIC_{CFDA} endpoints for all 12 of the low-high phenotype strains, when CFDA was incorporated into the macrodilution method (Fig. 4.4A, Fig. 4.4B). These findings provided further support for the *in vivo* evidence that has shown that the *C. albicans* low-high phenotype strains are susceptible to fluconazole (3, 27).

The culturing of inocula is normally reserved for determining of the minimum fungicidal concentration values with fungicidal agents (19). However, plate count data was included to assess the replication competency of the low-high phenotype strains (Fig. 4.4C). Azole antifungal agents are known to affect the development of hyphae in susceptible strains of *C. albicans* at doses that result in only a relatively small degree of inhibition of the growth rate (23). The plate counts of the low-high phenotype strains increased from 12 to 24 hr across the entire concentration range but remained the same at 24 hr and 48 hr (Fig. 4.4C). A reduction in plate counts of ≥80 % at 12, 24, and 48 hr coincided with the MIC_{ATP} endpoints at 12, 24, and 48 hr; the MIC_{CFDA}

endpoints at 12, 24, and 48 hr; and the $MIC_{reference}$ determined with a visual estimation of an 80% inhibition of growth at 24 hr. These observations confirm that the determination of a susceptible fluconazole MIC at 24 hr using the $MIC_{reference}$ method with a visual estimation of inhibition for the low-high phenotype strains is correct. The appearance of increased growth at higher concentrations of azoles does not correlate to increased CFUs. This suggests that either the trailing effect has an involvement with multicellular hyphae or is likely to be an artifact of the methodology.

Previous work has shown that exposing of *C. albicans* to subinhibitory concentrations of amphotericin B using a macrodilution based method produced higher levels of ATP than the drug-free control cultures (22). However, the authors measured total ATP without the selective evaluation of intracellular ATP levels. Amphotericin B binding to the fungal cell membrane alters its permeability and causes cytoplasmic constituents to leak out (20). Consequently, the accumulation of extracellular ATP was hypothesized to be due to the increased cell membrane permeability produced from the subinhibitory amphotericin B concentrations (2, 22).

We have previously shown that the concentration of intracellular ATP compared well with plate counts for the evaluation of *C. albicans* treated

with amphotericin B in a time-kill curve procedure (15). The work reported here shows that increasing concentrations of amphotericin B have a clear dose-response effect on the intracellular concentration of ATP for 12 strains of *C. albicans* using the NCCLS macrodilution method (21). The MIC_{ATP} endpoints had 100% agreement with those obtained using the $MIC_{reference}$ method at both 24 and 48 hr. MIC_{ATP} endpoints were possible at 30 min and 6 hr due to the rapid dose-responsive decrease in *C. albicans* intracellular ATP with amphotericin B exposure (Fig. 4.5). The observation that there was a 75% and 100% essential agreement between the $MIC_{reference}$ endpoints at 48 hr and MIC_{ATP} endpoints at 30 min and 6 hr, respectively, reflects the limit of the time required for amphotericin B to decrease intracellular ATP concentration. Further work will have to be done to improve the MIC_{ATP} method to decrease the time required for amphotericin B susceptibility testing to below 6 hours.

In conclusion, we have shown that measuring *C. albicans* intracellular ATP has considerable promise as a rapid method for obtaining amphotericin B susceptibility results. The MIC_{ATP} method agreed well with the NCCLS macrodilution method (21) after incubation for 12 hr with fluconazole and 6 hr with amphotericin B. In addition the MIC_{ATP} method confirmed the previous *in vitro* findings that the low-high

phenotype strains were susceptible using a CFDA-modified microdilution method (16). Both the MIC_{ATP} and MIC_{CFDA} methods demonstrate the potential of utilizing measurements that directly assess parameters associated with *C. albicans* viability (15), to expedite meaningful MIC data that correlate well with known *in vivo* results.

V. FIGURES

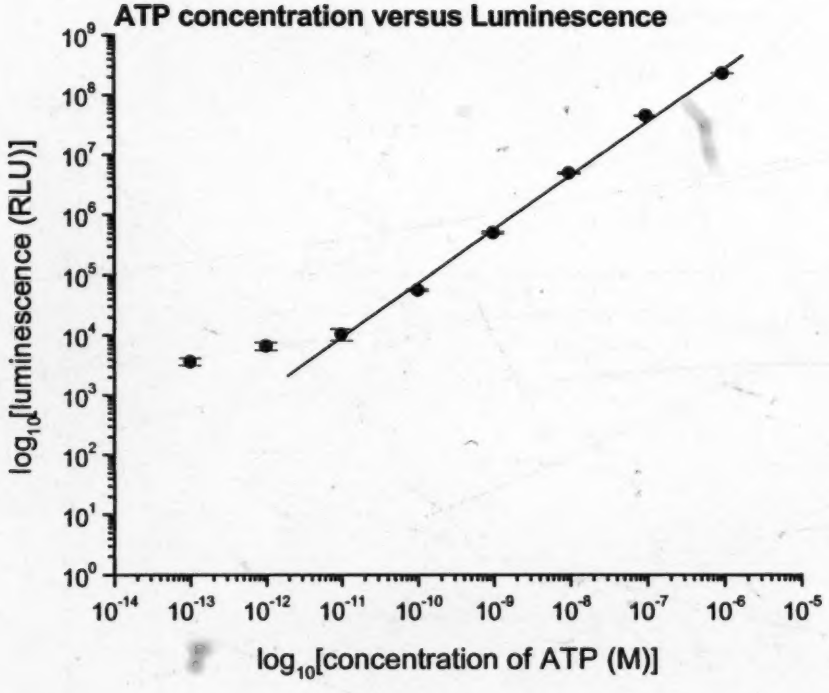

FIG. 4.1. Standard curve for the luciferase ATP assay with a boiling Tris EDTA extraction procedure. Relative luminescent units versus ATP concentration (M).

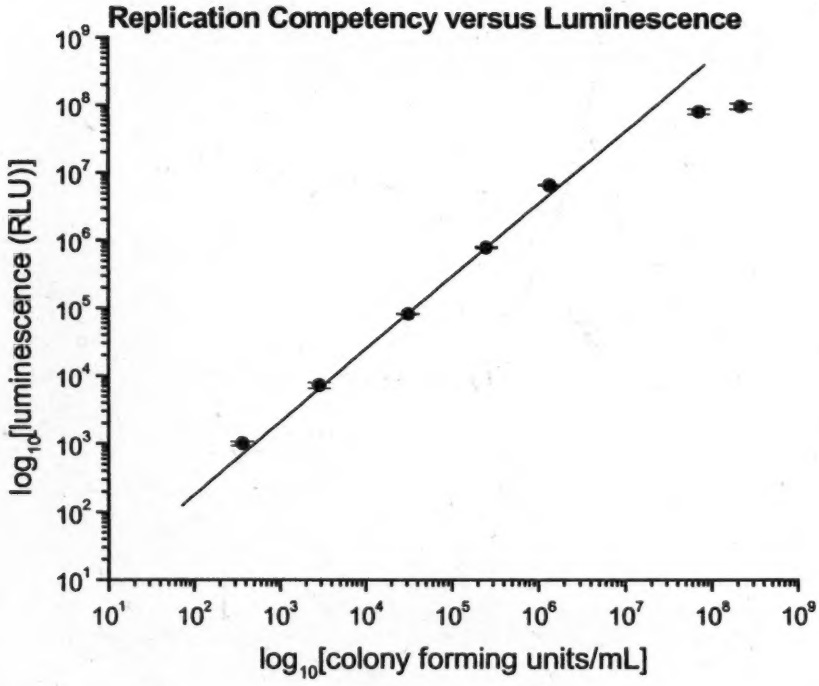

FIG. 4.2. Correlation between ATP relative luminescence units and replication competency as determined by plate counts (CFU/mL) for the growth of *C. albicans* strain ATCC 90028.

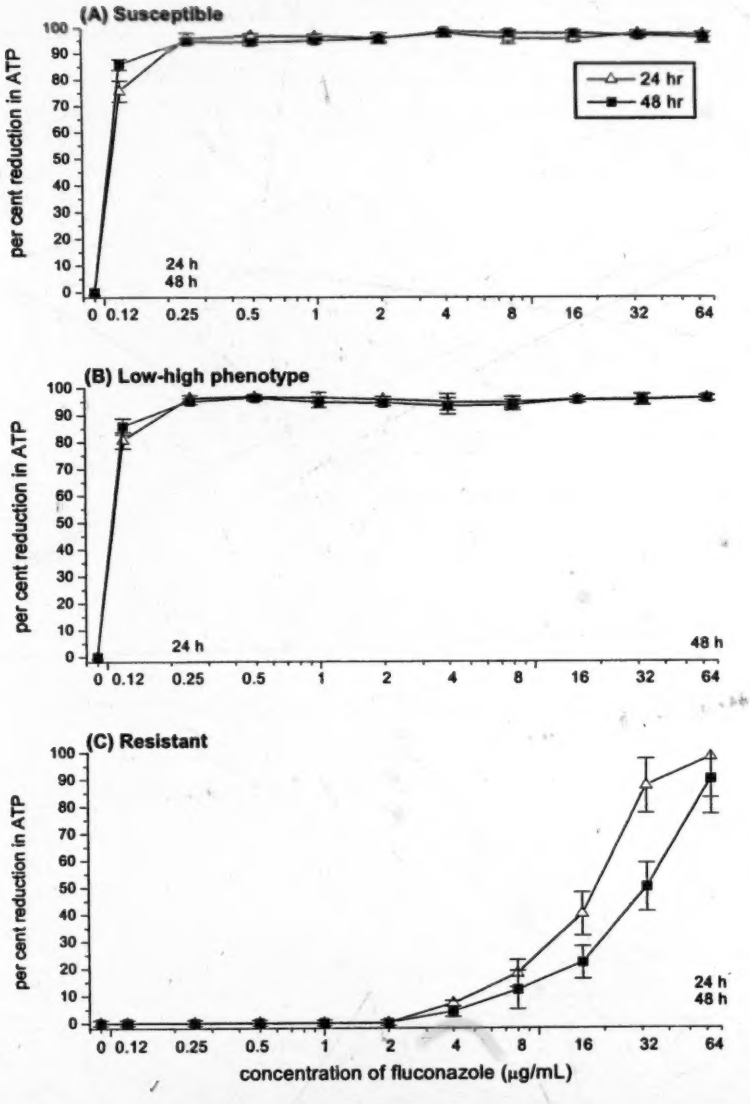
(A) Susceptible
per cent reduction in ATP
24 hr
48 hr
24 h
48 h
0 0.12 0.25 0.5 1 2 4 8 16 32 64
(B) Low-high phenotype
per cent reduction in ATP
24 h
48 h
0 0.12 0.25 0.5 1 2 4 8 16 32 64
(C) Resistant
per cent reduction in ATP
24 h
48 h
0 0.12 0.25 0.5 1 2 4 8 16 32 64
concentration of fluconazole (μg/mL)

Fig. 4.3. Effect of fluconazole on representative strains of *C. albicans* at 24 and 48 hr. Using the NCCLS macrodilution method (21): strain Y91 was susceptible with an MIC of 0.25 μg/mL at 24 and 48 hr (A), strain MY894 was low-high phenotype with an MIC of 0.25 μg/mL at 24 and 64 μg/mL at 48 hr (B), and strain 965 was resistant with an MIC of 64 μg/mL at 24 and 48 hr (C). The results of the intracellular ATP susceptibility test (MIC_{ATP}) are shown graphically as per cent reduction. The intracellular ATP content of the drug-free control was defined as 100% and the fluconazole-exposed tubes were scaled to this value. The MIC_{ATP} endpoint for FLU susceptibility testing was defined as the lowest concentration of antifungal drug that had ≥90% decrease in intracellular ATP. 24 and 48 represent the MICs determined at 24 and 48 hr respectively using the reference method.

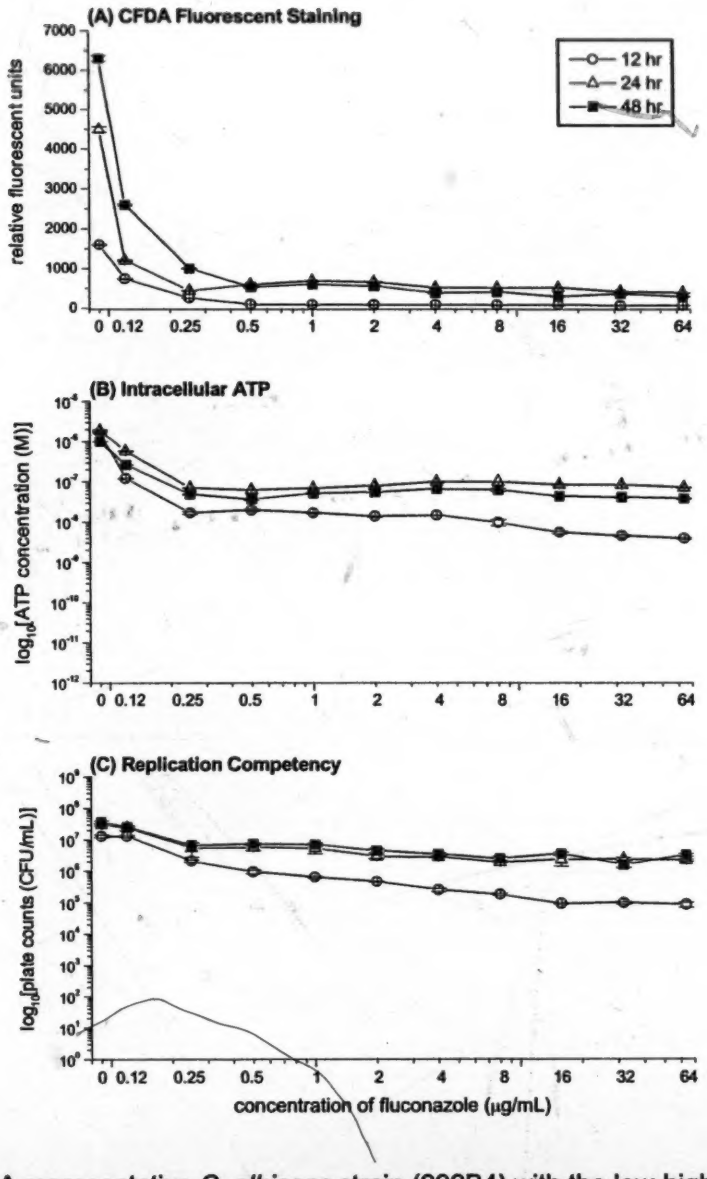

Fig. 4.4. A representative *C. albicans* strain (992B4) with the low-high phenotype incubated with fluconazole for 12, 24, and 48 h using the NCCLS macrodilution method (21). The susceptibility to fluconazole was evaluated with CFDA using an endpoint of ≥80% inhibition (A), intracellular ATP concentration using an endpoint of ≥90% inhibition (B), and replication competency measured as CFU/mL (C). The MIC determined using the reference macrodilution for 992B4 was 0.25 μg/mL at 24 hr and 64 μg/mL at 48 hr (not shown). The MICs determined using the MIC_{ATP} and MIC_{CFDA} methods were 0.25 μ g/mL at 6 hr and 48 hr. The y-axis for intracellular ATP (C) is scaled from 10^{-5} to 10^{-12} and reflects the limit of the measurable range of ATP using the described assay (Fig. 4.1). Error bars indicate standard error.

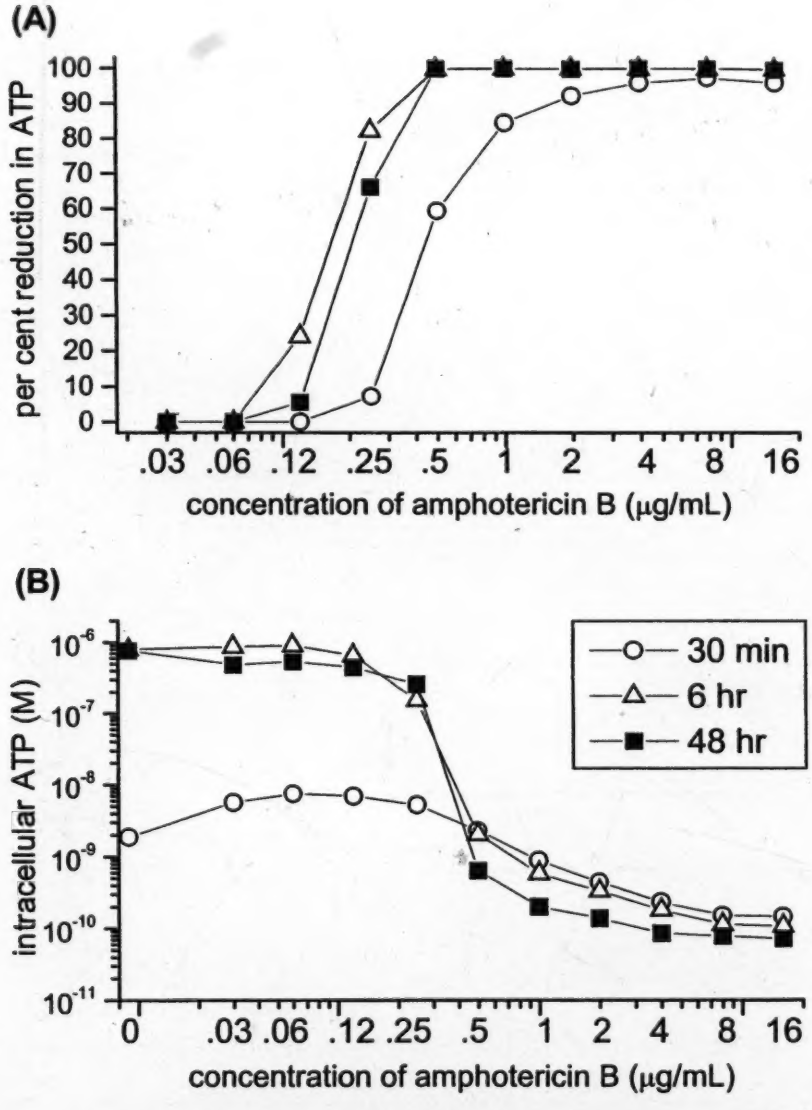

Fig. 4.5. Effect of amphotericin B on the intracellular ATP concentration for *C. albicans* strain ATCC 24433 using the NCCLS macrodilution method (21). The data is shown with exposure times of 30 min, 6 hr, and 48 hr as the per cent reduction in ATP (A) and the actual concentration of ATP (B). The MIC that was determined using the reference macrodilution method with a visual endpoint of 100% inhibition was 0.25 μ g/mL at 24 hr and 0.5 μ g/mL at 48 hr. The MIC that was determined by measuring the intracellular ATP concentration (MIC_{ATP}) with an endpoint of ≥99% inhibition was 0.5 μ g/mL at 24 and 48 hr. The NCCLS reference MIC range for ATCC 24433 was 0.25-1.0 μ g/mL (18).

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Chapter 5

Assessment of the Effect of Amphotericin B on the Vitality and Mortality of *Candida albicans*

Adapted from the original publication:

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I. INTRODUCTION

Candida albicans is both a commensal organism and opportunistic pathogen of humans. Morbidity and mortality associated with systemic infections caused by *C. albicans* remain unacceptably high because of difficulties in diagnosis and treatment (11). A mainstay of treatment for patients with invasive mycoses is the polyene macrolide antifungal amphotericin B (AMB). AMB binds to ergosterol, the principal sterol in the fungal cytoplasmic membrane. AMB molecules are believed to insert into the fungal cytoplasmic membrane and form pore-like structures, which culminate in osmotic instability, loss of membrane integrity, and metabolic disruption (4, 6).

Antifungal susceptibility testing remains dependent on the enumeration of replication-competent yeast cells with long incubation times and semiquantitative and subjective endpoints (11, 22, 28). Better, direct methods are required to evaluate yeast viability and the processes of fungal cell death and replicative deactivation to further our understanding of fungus-drug interactions. For example, the process of cell replication deactivation as envisaged by Jones (16) involves a stepwise change in the physiochemical state of a cell which renders an intermediate form incapable of initiating replicative processes but still

capable of metabolism. We wanted to evaluate cell death in *C. albicans* with amphotericin B to test if a more complex interaction was taking place than could be discerned only by estimating viability (ie. colony forming units). We used fluorescent dyes with specific cellular affinities to measure qualitative and quantitative characteristics essential to fungal cell viability (8, 13, 25). In combination, these vitality- and mortality-specific dyes monitor several physiological processes, such as membrane integrity, monitored with the fluorescent intercalating dye SYBR Green I, intracellular enzyme activity, monitored with the fluorogenic substrate 5,(6)-carboxyfluorescein diacetate (CFDA), and alterations in membrane potential, monitored with the fluorescent potentiometric probes bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] and 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$]. AMB-treated *C. albicans* was investigated by comparing the levels of fluorescence from the four different probes to a standard time-kill curve. The results are consistent with the presence of four different phenotypic states that are dependent on the concentration of AMB and the exposure time.

II. MATERIALS AND METHODS

Culture conditions and kill curve. *C. albicans* from frozen stock cultures was subcultured twice on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI) prior to use. The yeast strains were grown aerobically in yeast-peptone-dextrose (YPD) broth (1% mycological peptone, 1% yeast extract, 3% D-glucose) on a rotary shaker at 35°C for 10 to 12 h until the desired concentration of ~4×10^6 cells/mL (confirmed by plate counts) was obtained. A total of 100 mL of culture was decanted into 500-mL Erlenmeyer flasks, and the appropriate concentration of AMB (Appendix I) was added from a -75°C stock of 10,000 µg/mL in dimethyl sulfoxide (DMSO). The cells in these cultures were present as blastoconidia as confirmed by light microscopy. The culture flasks containing a range of AMB concentrations were then returned to incubation at 35°C in the dark. Each experiment included a control culture that was not exposed to AMB. A control culture that evaluated the effect of DMSO was also performed. One milliliter samples were taken from each incubating culture flask at 1.5, 4.5, and 10 h to quantitate intracellular ATP, total number of cells per milliliter, CFU per milliliter, and vitality- and mortality-specific dye fluorescence.

Particle counts. A 1 mL culture sample was centrifuged at 9,300 × *g* for 5 min at 25°C and resuspended in 1 mL of 0.1 M MOPS (3-[morpholino]propanosulfonic acid-sodium) (pH 7.0). The number of cells per milliliter in the sample was assayed with a Coulter M430 Counter (Coulter Electronics Inc., Hialeah, FL).

Scanning electron microscopy preparation. After centrifugation, the samples were washed with isotonic buffer fixed in 0.5% glutaraldehyde solution for 45 min and then washed again with isotonic buffer. The specimens were dehydrated in ethanol, coated with gold to a thickness of less than 20 nm and examined with a scanning electron microscope using an accelerating voltage of 2.0 kV.

Vitality- and mortality-specific fluorescent dyes. Fluorescent dyes were added after incubating the cultures in AMB for 1.5, 4.5, and 10 h, which eliminated uncertainties concerning growth-inhibiting effects of the staining process and also enhanced the ability to detect fungistatic activity (13). Pilot experiments established the most effective dye concentration, incubation time, temperature, pH, and number of wash steps. Details for each dye are summarized below. All samples were initially centrifuged at 9,300 × *g* for 5 min at the time of sampling. The

stained culture samples were divided into aliquots (200 µL per well) in triplicate in a 96-well Nunc-Immuno PolySorp plate (Nunc, Nalge Nunc International, Rochester, NY) and assayed for relative fluorescence intensity (RFU) with a FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT). All the fluorescent dyes could be optimally evaluated by using excitation and emission wavelengths of 485 and 530 nm, respectively. Stained cells were evaluated qualitatively by fluorescence microscopy.

(i) CFDA treatment. *C. albicans* cells were resuspended in MOPS buffer (0.1 M MOPS, pH 7), washed two more times, and resuspended a final time in MOPS buffer (pH 3) plus 50 mM citric acid. Ten microliters of 5 mg/mL CFDA (Sigma Chemical Co., St. Louis, MO) in DMSO was added to each 1 mL sample for a final concentration of 50 µg/mL. Samples were incubated with the stain in the dark at 35°C, with shaking, for 45 min. No additional wash step was then required.

(ii) $DiOC_6(3)$ treatment. *C. albicans* cells were resuspended in MOPS buffer (pH 10). 25.6 µL of 20 µg/mL $DiOC_6(3)$ (Sigma Chemical Co.) in DMSO was added to each 1 mL sample for a final concentration of 0.5 µg/mL. Samples were incubated with the stain in the dark at room

temperature for 30 min, then diluted 1:30 with Nonidet P-40 detergent (Sigma Chemical Co.) and incubated for 1 h with shaking in the dark at room temperature. Samples were then washed twice by centrifugation at 9,300 × *g* for 5 min and the resulting pellets were resuspended in MOPS buffer (pH 7.0). Samples were maintained on ice.

(iii) $DiBAC_4(3)$ treatment. *C. albicans* cells were resuspended in MOPS buffer (pH 7.0). Two microliters of 1 mg/mL $DiBAC_4(3)$ (Molecular Probes Inc., Eugene, OR) in 100% ethanol was added to each 1 mL sample for a final concentration of 2 µg/mL. Incubation with the stain was performed in the dark at room temperature with shaking for 1 h. The samples were then washed in MOPS buffer (pH 7.0) two times, as described above. Samples were maintained on ice.

(iv) SYBR Green I treatment. *C. albicans* cells were resuspended in MOPS buffer (pH 7.0). Fifteen microliters of a 1:100 diluted stock of SYBR Green I (14) in MOPS buffer (pH 7.0) was added to each 1 mL sample. Incubation with the stain was performed in the dark on ice at 4°C for 1 h. The samples were then washed two times in MOPS buffer (pH 7.0), as described above. Samples were maintained on ice.

Bioluminescence assay of ATP. The luciferase ATP assay was used to assay the effect of AMB on viable *C. albicans* cell biomass (24). The viable-cell count of *C. albicans* exposed to an antifungal agent has been shown to be directly related to intracellular ATP levels (2). ATP was assayed by measuring luminescence produced by the oxidation of luciferin in the presence of luciferase and ATP.

(i) Analytical equipment and reagents. Light emission from the bioluminescence assay was measured in a Bio-Orbit (Turku, Finland) 1258 microplate luminometer. The luminescence reaction temperature was set internally to 21°C. The ATP assay mix (Sigma Chemical Co.) containing luciferin and luciferase was prepared fresh according to the manufacturer. Apyrase (purified grade I; Sigma Chemical Co.) was used to eliminate extracellular ATP before extracting intracellular ATP.

(ii) Elimination of extracellular ATP. A culture sample of 1 mL was centrifuged at 9,300 × *g* and the resulting pellet was resuspended in Tris-EDTA buffer (0.1 M Tris buffer [pH 7.8] containing 2 mM EDTA). Fifty microliters of the washed sample was incubated for 15 min at 37°C with 50 µL of 0.04% apyrase ATPase.

(iii) Extraction of intracellular ATP. After eliminating the extracellular ATP, 50 µL of the apyrase-treated sample was pipetted into 500 µL of boiling Tris-EDTA buffer. After boiling for 90 s, the extracts were cooled and frozen at -75°C for later analysis.

(iv) Luciferase ATP assay. The ATP assay mix (80 µL) was added to 200 µL of each thawed sample extract (in triplicate) in a nonstandard 96-well opaque plate (Corning Costar Corp., Cambridge, Mass.), and the intensity of the luminescence was determined for 10 s after 1 min of incubation. The ATP concentration present in the sample extracts was determined by reference to an ATP standard curve. ATP added to the extracts was used as an internal standard to correct for inhibition of the luciferase reaction. Correction for machine background luminescence was made by direct subtraction.

III. RESULTS

Replication competency and cell counts. Increasing AMB concentration resulted in a dose-dependent reduction of replication competency for as measured by agar plate counts (CFUs) *C. albicans* to

a maximum effect with 4 µg/mL at 10 h of incubation (Fig. 5.1, Fig. 5.2A). AMB concentrations higher than 4 µg/mL did not increase the extent or rate of killing. The number of yeast cells present in cultures exposed to AMB as measured by particle counts (Fig. 5.1, Fig 5.2A) did not decrease below that present at the time of culture inoculation (t_0). An increase in the particle counts after t_0 for cells exposed to between 0 and 0.3 µg of AMB per mL coincided with growing cultures with dividing cells (Fig. 5.2A). Replication competency and intracellular ATP content both decreased below t_0 levels in cultures exposed to AMB concentrations greater than or equal to 0.5 µg/mL (Fig. 5.2A and B). Beyond 4.5 h, there was no significant additional decrease in replication competency in response to AMB (Fig. 5.2C). Growth (CFU per milliliter), albeit suboptimal in comparison to that of the control culture, was shown to occur at 4.5 and 10 h for cultures exposed to concentrations of AMB up to 0.3 µg/mL. *C. albicans* culture exposed to 0.4 µg of AMB per mL showed no significant increase or decrease in replication competency over the time course. A concentration of 0.4 µg of AMB per mL was thus fungistatic. A decrease in agar plate counts indicative of 99% inhibition of growth for *C. albicans* at 10 h occurred at 0.5 µg/mL (Fig. 5.2A), which corresponded to (i) the predetermined MIC and (ii) the lowest AMB

concentration shown to be capable of causing a decrease in CFU per milliliter at 10 h (Fig. 5.2C).

Intracellular ATP. The decrease in intracellular ATP at 10 h reached a plateau at an AMB concentration of 1.5 μg/mL and was paralleled by a decrease in replication competency (Fig. 5.2A and B). The lowest AMB concentration tested that resulted in a 99% reduction of intracellular ATP concentration after 10 h of incubation was defined as the AMB minimum effective concentration and was determined to be 0.2 μg/mL. The detection limit for detecting intracellular ATP with this assay was 10^{-10} M.

Vitality-specific fluorescent staining. Fluorescent staining of AMB-treated *C. albicans* 96-90 with CFDA and $DiOC_6(3)$ (Fig. 5.3A) showed an exponential decrease in RFU in a dose-dependent manner, plateauing at a concentration of 0.5 μg of AMB per mL. The other two strains behaved similarly (data not shown). The decreased fluorescence intensity presumably corresponded to decreased intracellular sequestration of dye within the cell (CFDA) or a decreased membrane binding [$DiOC_6(3)$].

Mortality-specific fluorescent staining. (i) SYBR Green I. *C. albicans* 96-90 cells stained with SYBR Green I showed a linear increase in RFU with increased exposure to AMB at concentrations between 2.0 and 4.0 µg/mL, with a plateau in RFU between 0.4 and 2.0 µg/mL and a peak in RFU at 0.2 µg/mL (Fig. 5.3B). SYBR Green I mortality-specific staining showed a gradual increase in staining from 1.5 to 10 h for cultures exposed to greater than 2.0 µg of AMB per mL, with RFU values at an AMB incubation time of 4.5 h intermediate to those at 1.5 and 10 h (Fig. 5.4A). The peak in RFU present in the culture incubated with 0.2 µg of AMB per mL was shown to occur at 10 h of incubation and not earlier. SYBR Green I staining was similar for the other two *C. albicans* strains tested.

(ii) $DiBAC_4(3)$. Fluorescent staining of *C. albicans* 96-90 with $DiBAC_4(3)$ showed a linear increase in RFU in cell cultures incubated with increasing AMB concentrations between 0.8 and 4.0 µg/mL (Fig. 5.3B). This increase occurred at incubation times greater or equal to 4.5 h (Fig. 5.4B). At 1.5 h of exposure to AMB, mortality-specific staining did not increase below a concentration of 2.0 µg/mL. A peak in $DiBAC_4(3)$-specific staining occurred in cultures exposed to 0.2 and 0.3 µg of AMB per mL, which coincided with the cultures which grew during AMB

exposure. This peak in fluorescence was present at 10 h but not at the early times of 1.5 or 4.5 h (Fig. 5.4B). $DiBAC_4(3)$ staining showed a similar pattern for the other two *C. albicans* strains tested (data not shown).

(iii) Replication competency and mortality-specific staining comparison. Over the time course of *C. albicans* culture incubation with AMB, the greatest mortality-specific fluorescent staining occurred after 10 h and only with cultures which had a significant reduction in replication competency. A direct graphical comparison of these results accentuates the observation that these cells, after 10 h of exposure to AMB at concentrations between 0.5 and 1.0 µg/mL, are not able to replicate on agar plates but do not take up mortality-specific dyes (Fig. 5.5).

IV. DISCUSSION

Yeast viability is a measure of the number of living cells, whereas vitality can be seen as a function of the total cell viability and the physiological state of that population (18). Difficulties in quantifying

microbial killing are due in large part to which properties are attributed to the state of being alive, since the presence of dead microbes must be inferred retrospectively from estimates of these properties. For instance, reproductive competency as established through plate counts has long been the property considered to be the "gold standard" for cellular viability, even though it is recognized that only a fraction of viable cells replicate when stressed (21) and equates cell division with viability which may be inaccurate.

Direct measurements of yeast cell vitality and mortality would provide a better understanding of antifungal activity and perhaps lead to advances in design of antifungals. Vitality- and mortality-specific fluorescent dyes, interpreted jointly, can distinguish not only between live and dead microorganisms but also between the "vigorous, frail and injured" (19), potentially providing a summary evaluation of the true viability of each subpopulation affected by the drug. Microbiologists attempting to describe the physiological state of microbes often divide them into the categories of alive and dead. Alive and dead represent extremes along the continuum.

The number of *C. albicans* cells, as detected by particle count, present in the AMB-exposed cultures did not decrease below that initially present at the time of culture inoculation, suggesting that the cells, while

incapable of replication on agar plates, were still intact (Fig. 5.2A). This was confirmed directly with scanning electron microscopy. Observations with scanning electron microscopy confirmed the presence of intact cells (Fig. 5.6 and 5.7). The debris present in figure 5.7 was believed to be material from the exterior of the *C. albicans* cell wall. This exterior cell wall material is likely made up mannan glycoproteins and is present on the surface of the growing control cells (Fig. 5.6B) but is absent on the surface of cells exposed to high concentrations of AMB for 10 hr (Fig. 5.7B). Depending on their physiologic state, intact cells are able to take up and bind fluorescent dyes.

Metabolic pathways can be stoichiometrically related through the adenine nucleotide system (9), and thus intracellular ATP was used as a measure of *C. albicans* cell metabolic potential and was shown to decrease in a dose-dependent manner with increasing AMB concentration (Fig. 5.2B). The lowest concentration of AMB that was inhibitory to replication and caused a decrease in intracellular ATP content was 0.5 µg/mL, which corresponded to both the predetermined MIC and the minimum concentration of AMB which showed a dose-dependent decrease in fluorescent staining with the vitality-specific dyes CFDA and $DiOC_6(3)$ (Fig. 5.3A). The saturation of the inhibitory effect of

AMB on intracellular ATP at 1.2 µg/mL AMB confirmed previous findings at 1.0 µg/mL AMB by Odds *et al.* (Odds 1985).

CFDA is a lipophilic, nonpolar substrate which traverses the cell membrane and is hydrolyzed by nonspecific intracellular esterases to the fluorescent anion carboxyfluorescein (27). Cells with compromised membranes rapidly leak carboxyfluorescein, even when residual esterase activity is retained intracellularly (15). $DiOC_6(3)$ is a lipophilic, cationic dye molecule that has an affinity for the negatively polarized membranes of living cells (20,26). AMB concentrations between 0 and 0.5 µg/mL reduced the vitality of *C. albicans* cultures as defined by esterase activity and membrane integrity (CFDA), electrochemical potential [$DiOC_6(3)$], and metabolic potential (ATP).

The AMB time-kill curves showed inhibition of replication competency (CFU per milliliter) with increasing concentrations of AMB between 0.5 and 4.0 µg/mL (Fig. 5.2A). Both of the fluorescent mortality-specific dyes, SYBR Green I and $DiBAC_4(3)$, showed increases in fluorescence for cultures exposed to increasing concentrations of AMB, reaching a maximum fluorescence at 4.0 µg of AMB per mL.

We observed several significant differences between the mortality-specific staining of SYBR Green I and that of $DiBAC_4(3)$. SYBR Green I is a nucleic acid-binding dye which increases in fluorescence after

intercalation into double-stranded DNA. Intact membranes thus exclude the dye and prevent binding to the DNA, and we assume that significant damage to the cell membrane must take place to allow access. SYBR Green I did not bind to DNA significantly until cultures were exposed to concentrations of AMB above 2.0 µg/mL for a full 10 h (Fig. 5.4A). Above this concentration, intracellular ATP concentration also decreased to a minimal value, which may also indicate extensive damage to the cell membrane (Fig. 5.2B). A concentration of 2 µg of AMB per mL may represent the threshold required to induce sufficient membrane damage to saturate the cellular repair mechanism if exposed for 10 hours. At incubation times of 1.5 and 4.5 h, even concentrations of AMB above 2.0 µg/mL did not result in significant uptake of SYBR Green I. Thus, binding of SYBR Green I was time- and concentration-dependent.

$DiBAC_4(3)$ (Fig. 5.4B) is an anionic lipophilic dye sensitive to membrane potential. Normal cells have a negative surface charge and thus exclude the dye. Damaged cells depolarize, allowing the dye to penetrate, bind to lipid-rich intracellular components, and fluoresce (3). The loss of membrane potential is thus inferred from increased $DiBAC_4(3)$ cellular staining (8, 10). Similar to the results obtained with SYBR Green I, the cultures exposed to AMB for 1.5 h showed increasing

dose-responsive staining only with AMB concentrations above 2.0 µg/mL. However, incubations of 4.5 and 10 h with AMB concentrations between 0.5 and 4.0 µg/mL resulted in dose-dependent increases in fluorescence. Thus, replication-incompetent cells that do not take up mortality-specific dyes can be induced to take them up with increased concentrations of AMB, increased incubation time, or a combination of the two. $DiBAC_4(3)$ detection of this transition requires at least 1.5 µg of AMB per mL for 4.5 h, and SYBR Green I detection requires at least 2 µg/mL for 10 h. It is reasonable to assume that while both of these populations are incapable of replication, they represent physiological states which are part of a viability continuum between alive and dead.

Attempts to define the stages of any developmental continuum is doomed to imperfection: first, each stage grows out of some previous stage and the lines of demarcation are inevitably arbitrary; second, any one stage of the continuum will have a heterogeneous population of cells. Nevertheless, arbitrarily delineated stages provide a useful shorthand for discussing the diversiy, provided one bears in mind the aforementioned caveats. These stages also acknowledge the complexity of microbial diversity and thus are superior to the conventional dead or alive polar definitions. With this understanding we developed a simple classification of the stages of yeast viability using

four categories. These four physiological states were defined using six different viability markers after exposure of *C. albicans* to increasing concentrations of AMB (Table 5.1 and Fig. 5.5). The described states represent a progression along the continuum from viability to death. Cells exposed to AMB at concentrations between 0.5 and 1.0 µg/mL do not show uptake of vitality- or mortality-specific dyes and are replication incompetent but may represent cells capable of resuscitation. The possibility of resuscitation and outgrowth of these replication-deficient *C. albicans* cells in a systemic infection could represent an important therapeutic problem, especially in an immunocompromised host. Postantifungal growth suppression with subsequent recovery of *C. albicans* has been shown previously with AMB at these concentrations and incubation times and may in part explain this phenomenon (29). Gale *et al.* (12) have also shown that *C. albicans* cultures entering the stationary phase of growth develop phenotypic resistance to AMB due to ultrastructural changes in the cell wall. Possible resuscitation or phenotypic resistance of these replication-incompetent cells, which do not show uptake with either vitality- or mortality-specific dyes, has not yet been determined.

With clinical use, peak serum concentrations of AMB with conventional intravenous doses are initially between 0.5 to 2.0 µg/mL, fall rapidly, and

then slowly reach a plateau between 0.2 and 0.5 µg/mL (5). It is interesting that *C. albicans* exposed to this concentration range was shown to retain viability, and this may help explain the need for prolonged treatment and frequent clinical failure with this drug.

Cultures exposed to AMB concentrations between 0.2 and 0.3 µg/mL for 10 h permitted suboptimal growth to occur (Fig. 5.2C), and an increase in mortality-specific dye fluorescence was observed (Fig. 5.4). This peak in fluorescence may be the result of an increase in the number of nonviable cells present in stationary phase at 10 h, which accompanies the increase in total cell number from t_0. Alternatively, the cytoplasmic membrane and/or cell wall of *C. albicans* synthesized during suboptimal growth in the presence of AMB concentrations between 0.2 and 0.3 µg/mL may have increased permeability and allowed access of the mortality-specific dye (17). Anomalous findings were previously reported with *C. albicans* exposed to low, sublethal AMB concentrations, including increased CFU (7), reduced adherence to surfaces and germ tube formation (23), and reduced synthesis of surface mannan (1).

It has been suggested that in cell populations under stress, an equilibrium exists whereby small disturbances are tolerated but extreme stress reduces vitality (19). This may result in a physiologic state where

true viability is retained but normal cellular functions, such as replication or the maintenance of membrane potential, are reduced (19).

Increasing exposure of *C. albicans* cells for 10 h to AMB concentrations up to 0.5 µg/mL results in greatly decreased uptake of vitality-specific dyes, decreased concentrations of intracellular ATP, and greatly reduced replication. The remaining cells show gradually decreasing membrane potential with AMB concentrations above 1.0 µg/mL and decreased membrane integrity at concentrations above 2.0 µg/mL. Above 4.0 µg/mL, increasing AMB concentration or incubation time did not result in further decreases in membrane potential or membrane integrity.

Comparison of the time-kill curve to the fluorescent dye data of the AMB-exposed *C. albicans* cells allowed for a separate assessment of replication competency, vitality, and mortality. Using these indicators, we were able to describe four different response categories of *C. albicans* to AMB, which represented a progressive spectrum of AMB-induced cell damage (Table 5.1). This demonstrates that the processes taking place during the exposure of *C. albicans* to AMB occur gradually and that the failure of replication is only one measurement of the process, which may lead to cell death. The work described here provides evidence for the utility and potential clinical importance of

evaluating mortality and vitality separately to develop an overall understanding of true viability. We believe these findings indicate that further investigation of the physiological changes of *C. albicans* in response to AMB exposure is warranted.

V. TABLES

Table 5.1. Summary of four physiological states of C. albicans as a consequence of incubation with increasing concentrations of amphotericin B (amb) for 10 h

Method of evaluation	Physiological parameter measured	Result at AMB conc. (μg/mL) of[a]:			
		0-0.5	0.5-1.0	1.0-2.0	2.0-4.0
Agar plate counts (<99% inhibition of control)	Replication competency	Yes	No	No	No
Intracellular ATP (<99% inhibition of control)	Metabolic potential	Yes	No	No	No
Vitality dyes					
$DiOC_6(3)$	Negative membrane potential	Yes	No	No	No
CFDA	Intracellular enzyme activity	Yes	No	No	No
Mortality dyes					
$DiBAC_4(3)$	Positive membrane potential	No	No	Yes	Yes
SYBR Green I	Loss of membrane integrity	No	No	No	Yes

[a] Yes, positive result for physiological parameter being measured; no, negative result for the physiological parameter being measured

VI. FIGURES

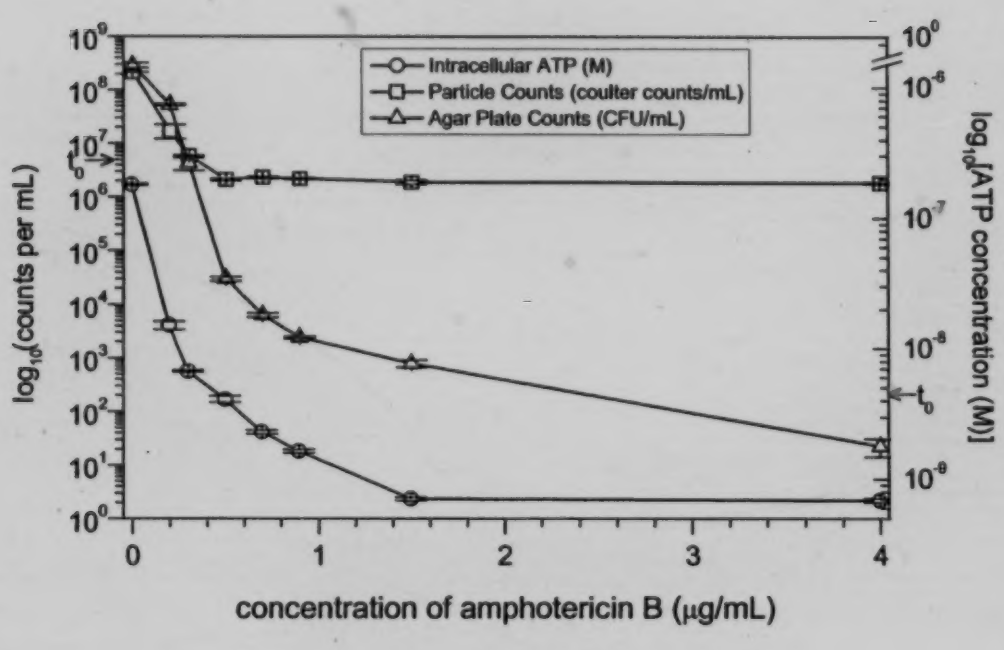

Fig. 5.1. Evaluation of *C. albicans* strain MY894 (AMB susceptible) after 10 h incubation with increasing concentrations of AMB. Replication competency determined by plate counts (CFU/mL) and particle counts determined with a coulter counter are shown on the y-axis labelled *counts per mL* and the concentration of intracellular ATP is shown on the other y-axis. t_0 represents a measurement of the *C. albicans* culture prior to incubation with AMB. Error bars indicate standard error.

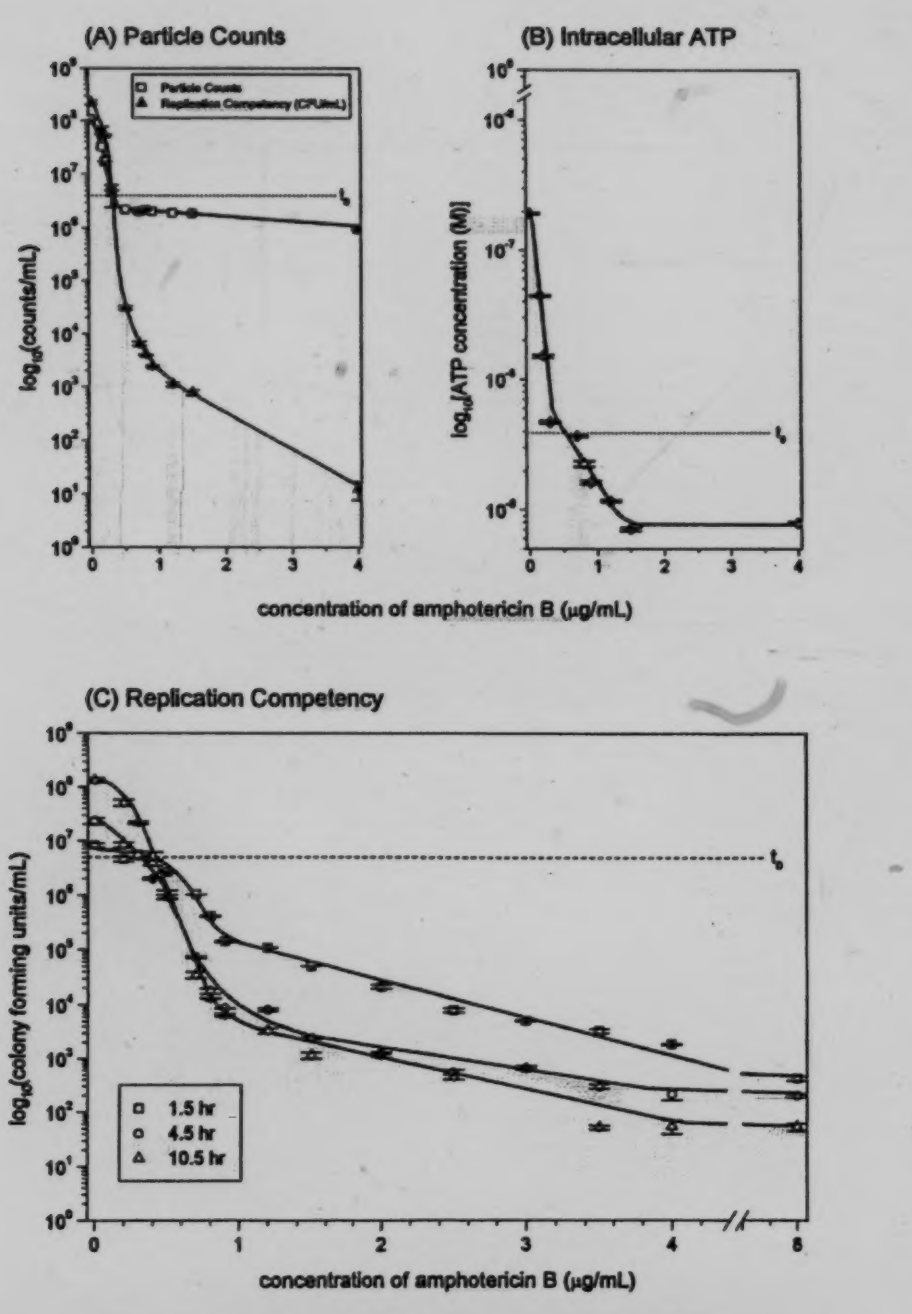

FIG. 5.2. Evaluation of *C. albicans* strain 96-90 incubation with increasing concentration of amphotericin B (AMB), showing (A) replication competency and particle counts at 10 hr, (B) intracellular ATP at 10 hr, and (C) replication competency at 1.5, 4.5 and 10 hr. t_0 represents a measurement of the *C. albicans* culture prior to incubation with AMB. Error bars, standard error.

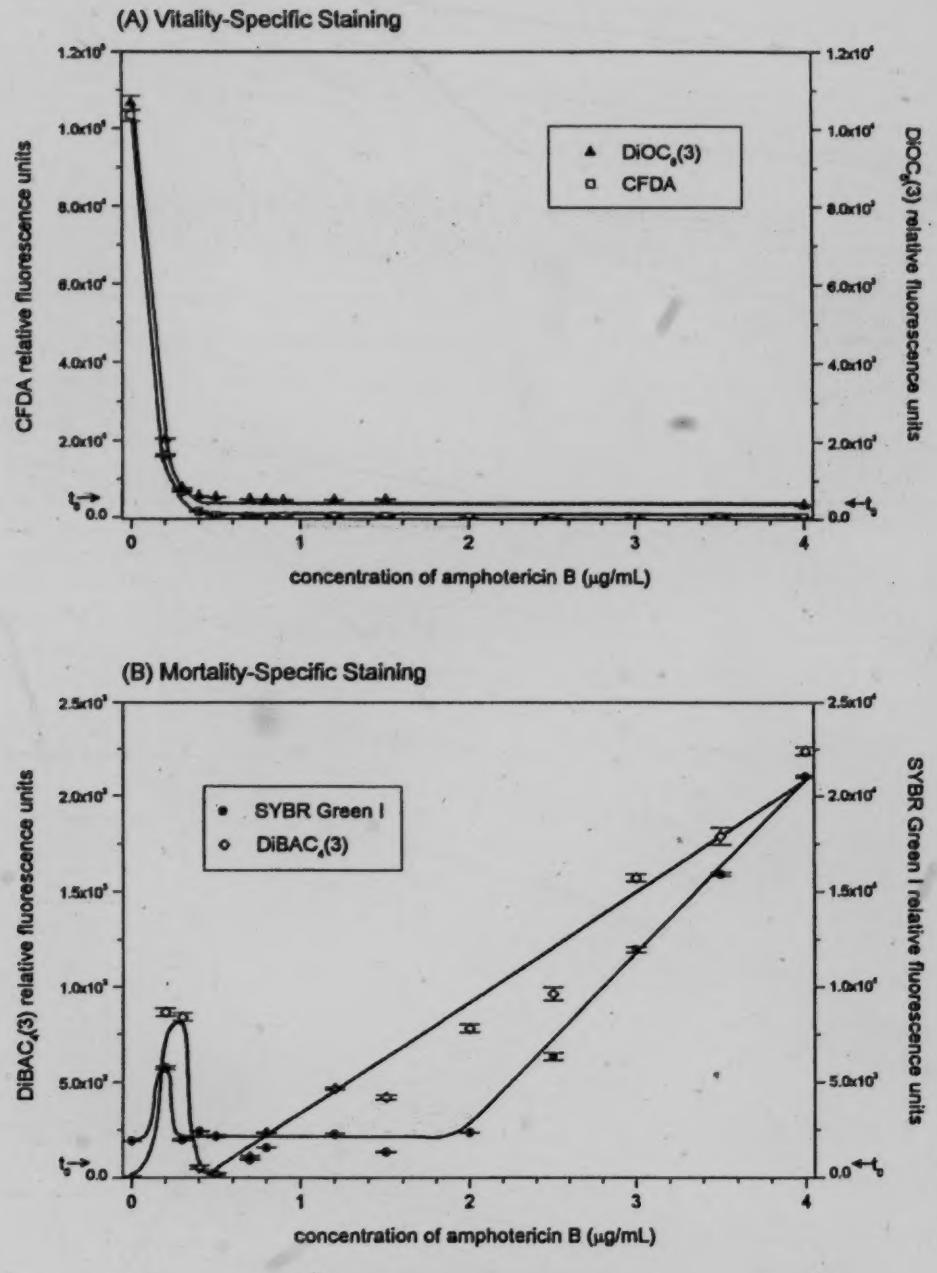

FIG. 5.3. *C. albicans* strain 96-90 incubation with AMB at 10 hr. Evaluation using (A) vitality-specific fluorescent staining ($DiOC_6(3)$ and CFDA) and (B) mortality-specific fluorescent staining ($DiBAC_4(3)$ and SYBR Green I). t_0 represents a measurement of the *C. albicans* culture prior to incubation with AMB. Error bars, standard error

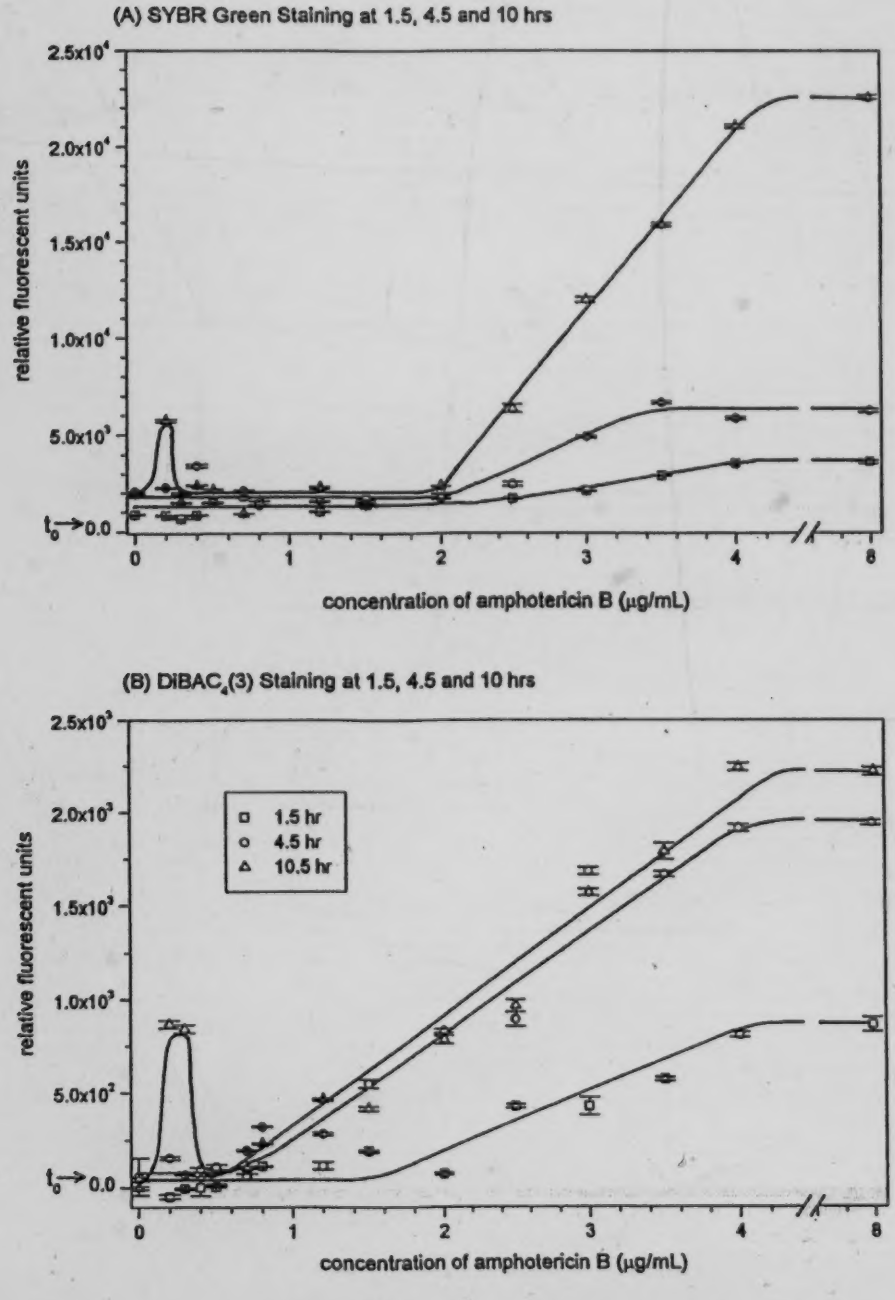

FIG. 5.4. Time-kill curves for *C. albicans* strain 96-90 with AMB at 1.5, 4.5 and 10 hr. as evaluated using mortality-specific staining. t_0 represents a measurement of the *C. albicans* culture prior to incubation with AMB. Error bars, standard error.

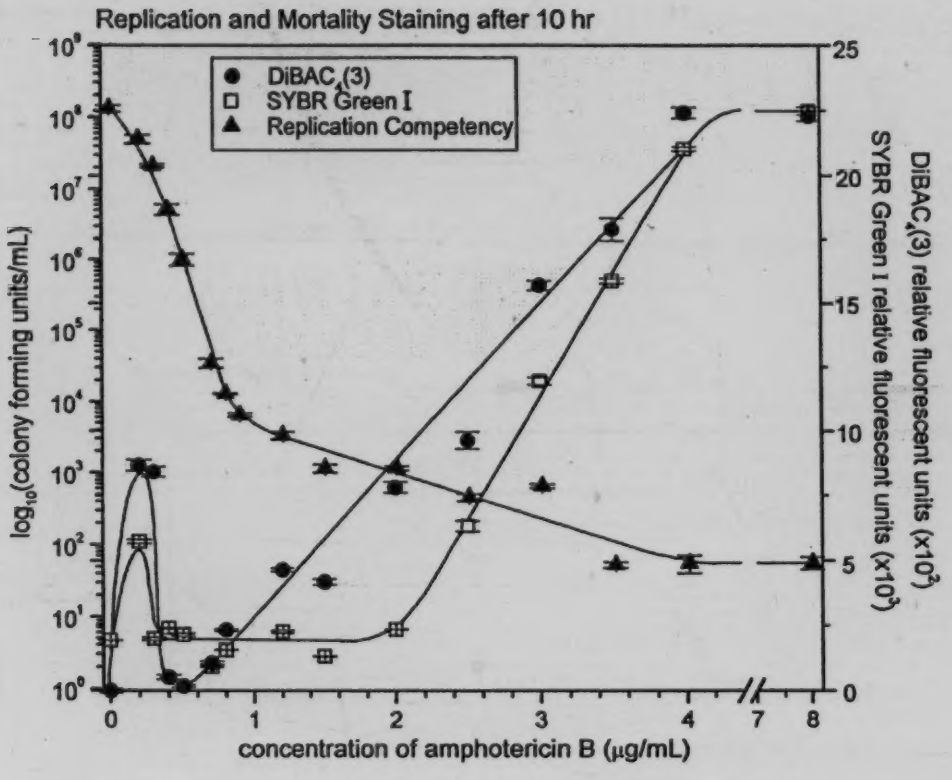
Replication and Mortality Staining after 10 hr
DiBAC4(3)
SYBR Green I
Replication Competency
log10(colony forming units/mL)
DiBAC4(3) relative fluorescent units (x10²)
SYBR Green I relative fluorescent units (x10³)
concentration of amphotericin B (μg/mL)

FIG. 5.5. Time-kill curve for *C. albicans* strain 96-90 with AMB at 10 hr as evaluated using replication competency and mortality-specific staining. Error bars, standard error.

Fig 5.6. Scanning electron micrograph of *C. albicans* 96-90 untreated growth control after 10 hr at 35°C. (A) Magnification 10,000×. Dotted bar represents 3 μM. (B) Magnification 30,000×. Dotted bar represents 1 μM.

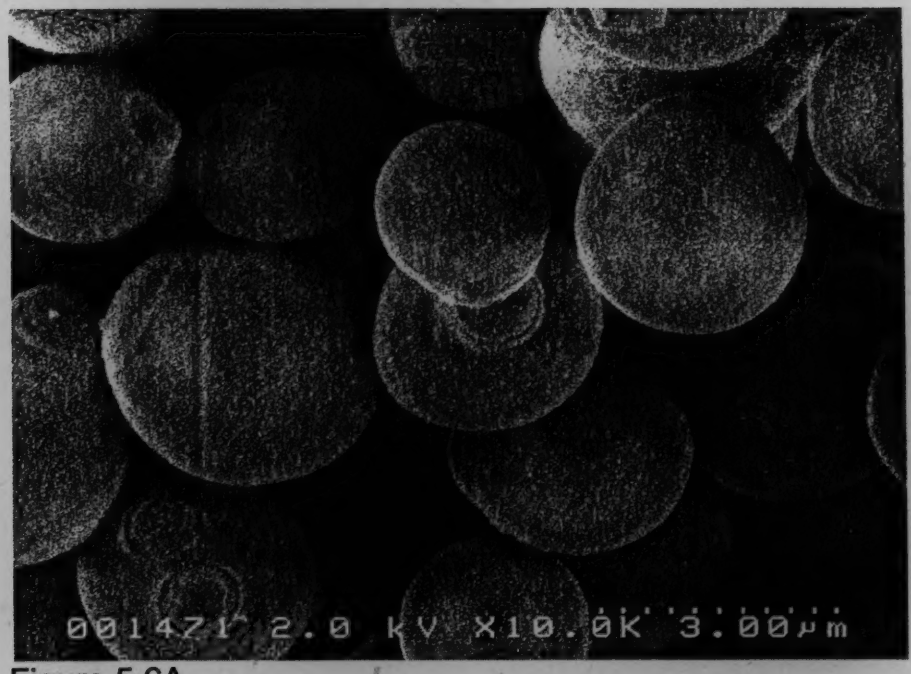

Figure 5.6A

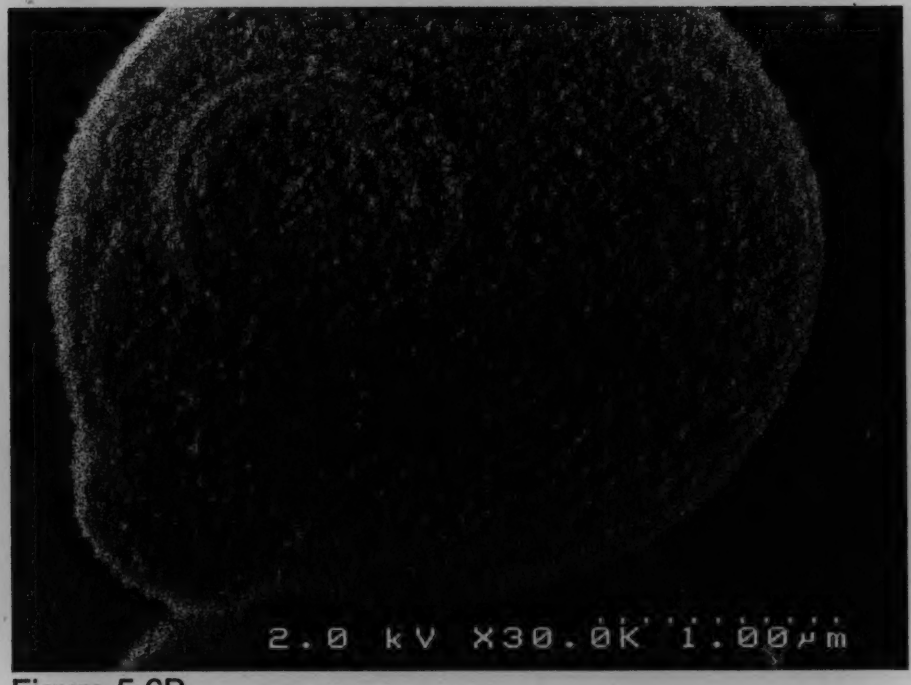

Figure 5.6B

Fig 5.7. Scanning electron micrograph of *C. albicans* 96-90 treated with 1.2 μg/mL of amphotericin B for 10 hr at 35°C. (A) Magnification 10,000×. Dotted bar represents 3 μM. (B) Magnification 30,000×. Dotted bar represents 1 μM.

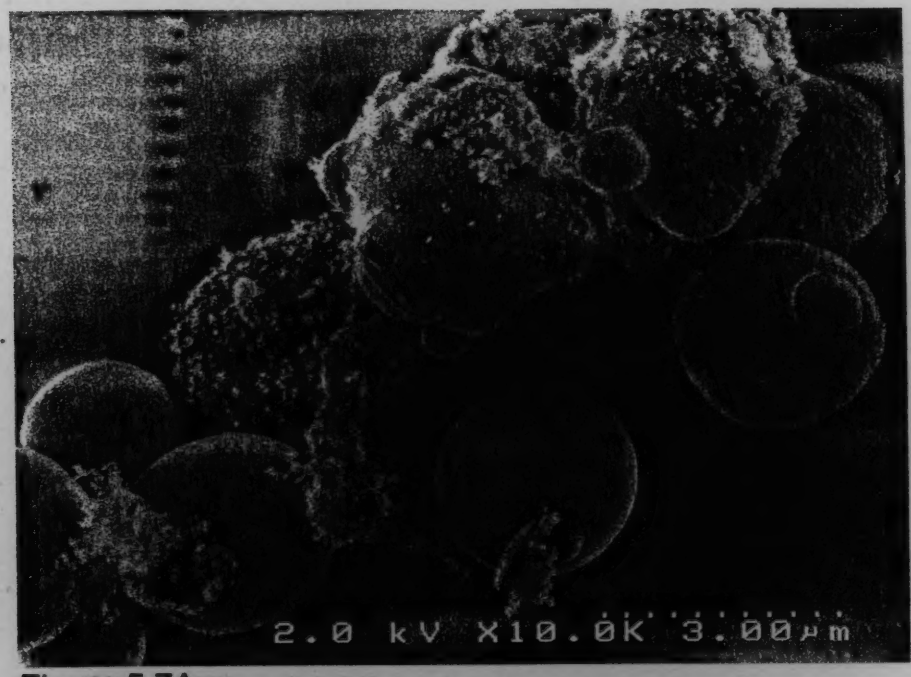

Figure 5.7A

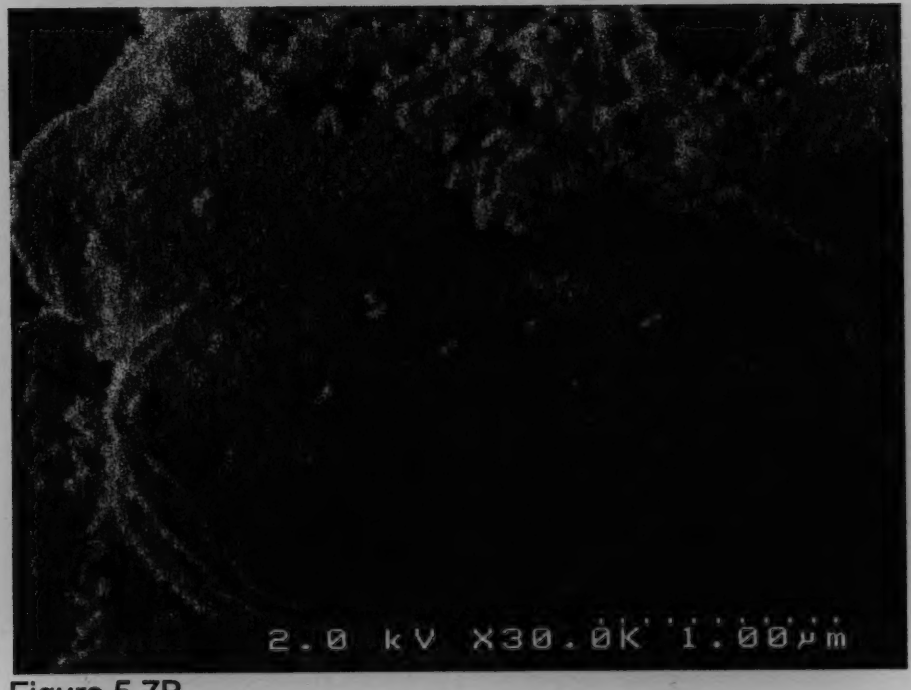

Figure 5.7B

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Chapter 6

Sublethal Injury with Resuscitation or Killing of *Candida albicans* after Amphotericin B Treatment

Adapted from the submitted manuscript (June 2002)

with requested minor revisions (July 2002)

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Antimicrobial Agents and Chemotherapy

I. INTRODUCTION

Candida is the fourth most common cause of nosocomial bloodstream infection in the United States (10) and the rate of primary bloodstream infections continues to increase (3). The most frequently isolated *Candida* species in these infections is *C. albicans*. Candidemia is also frequently refractory to therapy and the attributable mortality rate has been estimated to be 38% (36). Despite recent advances in antifungal agents, amphotericin B (AMB) remains the drug of choice for life-threatening systemic infections (14), and is the standard to which the activity of other antifungal agents are compared. AMB binds with the sterol ergosterol, resulting in the loss of membrane integrity and osmotic stability (2).

The process of cell replication deactivation is believed to involve stepwise changes in the physiological state of a cell that render an intermediate form incapable of initiating replicative processes but still capable of metabolism (21, 27). Thus, the loss of replication competency is an early event in a phase of decline that leads to eventual cell death (19, 27).

We have previously examined the effect of AMB on the vitality and mortality of *C. albicans* using the measurements of intracellular ATP and

fluorescent dyes with specific cellular affinities (22). This work delineated several physiological states produced as a consequence of incubating *C. albicans* with AMB for 10 hr. These states included alive, lethally injured, and a hypothesized state where the cells were sublethally injured because they did not stain with vitality- or mortality-specific fluorescent dyes but had a greater than 99% reduction in CFUs.

If the loss of replication competency is an early event in cell death it was postulated that the hypothesized sublethally injured state could be explained by a population of cells that could no longer reproduce but which remained metabolically active (22). Since it was shown that only a fraction of stressed organisms can be enumerated as CFUs on agar (23), this hypothesis seemed reasonable. If the cells are sublethally injured, it should be possible to demonstrate their metabolic activity, restore their ability to form colonies or convert the sublethal injury to a lethal one. We describe our further characterization of this interaction between *C. albicans* and AMB.

II. MATERIALS AND METHODS

Time-kill curves. (i) Initial 10 hr AMB treatment. The *C. albicans* strain was grown aerobically in yeast peptone dextrose broth (YPD; 1% mycological peptone, 1% yeast extract, 3% glucose) on a rotary shaker at 35°C until the desired concentration of approximately $4x10^6$ CFU/mL was obtained. A total of 100 mL of culture was decanted into 500 mL Erlenmeyer flasks and the various concentrations of AMB were added. The culture flasks containing a range of AMB concentrations from 0 to 4 μg/mL were then returned to incubation at 35°C in the dark with shaking for 10 h and assayed directly (1 mL samples) to quantitate the following: intracellular ATP concentration, CFUs per mL (CFU/mL), reduction of XTT, and the fluorescent intensity of staining with CFDA, $DiBAC_4(3)$ and SYBR Green I.

(ii) Sequential 15 hr retreatment. Following the 10 hr AMB treatment a representative number of these cultures (0, 0.5, 0.7, 0.9 and 4.0 μg/mL AMB) was further exposed to a battery of agents for an additional 15 hr at 21°C in the dark and assayed directly as before. These additional treatments included: (i) retreatment with the same concentration of AMB, (ii) 50 μg/mL fluconazole, (iii) 50 μg/mL itraconazole, (iv) 50 μg/mL ketoconazole, (v) 10 μg/mL miconazole, (vi)

20 μg/mL miconazole, (vii) 10 μg/mL 5-flucytosine, (viii) 100 μg/mL 5-flucytosine, and (ix) no additional treatment. These concentrations were chosen to achieve the maximum antifungal inhibition that could be expected with each respective antifungal agent.

XTT reduction. The assay is a modification of the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) measurements described by Tellier *et al.* (21). 1 mL of *C. albicans* culture was centrifuged at 9,300 x *g* for 5 min and then resuspended in RPMI-1640 modified medium without phenol red (Sigma, St. Louis, MO). The culture samples were initially incubated for 30 min at 35°C shaking at 160 rpm. A volume of 250 μL of a mix containing XTT (Sigma, St. Louis, MO) and menadione (Sigma, St. Louis, MO) were added to each 1 mL sample for final concentrations of 0.2 mg/mL of XTT and 1.25 μM of menadione. Menadione is an electron coupling agent that potentiates the reduction of XTT. The mix was made fresh with 8 mL of a 1 mg/mL stock of XTT in MOPS and 5 μL of a 10 mM menadione stock in acetone. The samples were incubated for 1 hr at 35°C to allow for colour development and then centrifuged as above. The colourimetric change of the supernatant solutions was measured at 470 nm. Dilutions were included as required.

III. RESULTS AND DISCUSSION

C. albicans MY2417 was exposed to a range of AMB concentrations from 0 to 4 μg/ml and the toxic injury that resulted was characterized by measuring seven different parameters (Fig. 6.1A, 6.1B, 6.1C, 6.1D). *C. albicans* grown for 10 hr at 37°C increased from $4x10^6$ particles (as measured with a Coulter Counter) and CFU per mL at the beginning of incubation to $9x10^7$ particles per mL and $1.7x10^8$ CFU per ml (Fig. 6.1A). Exposure to increasing concentrations of AMB inhibited this growth and *C. albicans* exposed to 0.3 μg/ml of AMB had no net growth over the 10 hr incubation period. At AMB concentrations above 0.3 μg/ml the number of particles remained stable at the same number as were present at time zero, but the organisms capable of forming colonies declined. There was a 90% and 99.9% reduction in CFUs at AMB concentrations of 0.4 and 0.5 μg/ml, respectively. At 1 μg/ml of AMB only $9x10^3$ of the organisms were still capable of reproduction. It has been the convention to refer to this as a fungicidal assay, assuming the failure to form colonies was due to fungal cell death (24). The reason for this assumption was that, when plating aliquots of antifungal-treated yeast to solid media, the diffusion of the antifungal agent into the buffer component of the medium reduced the concentration of the antifungal

so that yeast that were merely inhibited and not killed would resume reproduction and form colonies (1). Any cells that failed to grow are assumed to be dead (18). This assumption fails to consider antifungal agents with lengthy postantifungal effects (33), antifungal agents that do not quickly diffuse into the buffer medium e.g. lipophilic molecules already incorporated into the cell membrane, or injured organisms that are alive but incapable of replication.

Both, the intracellular ATP concentration (Fig. 6.1C) and the fluorescence produced by the vitality specific dye CFDA (Fig. 6.1B) decreased significantly to a plateau at approximately 0.3 μg/ml of AMB. CFDA is a nonpolar dye that is cleaved to carboxyfluorescein by nonspecific esterases in intact cells. In contrast, the *C. albicans* cultures stained with the mortality-specific dyes $DiBAC_4(3)$ and SYBR Green I produced only minimal levels of fluorescence until being exposed to concentrations of AMB above 0.6 μg/ml and 1.6 μg/ml, respectively (Fig. 6.1B). $DiBAC_4(3)$ is an anionic lipophilic dye sensitive to cytoplasmic membrane potential and excluded by normal cells with a negative internal charge (2). SYBR Green I is a nucleic acid binding dye that increases in fluorescence after intercalation into double-stranded DNA and is excluded by intact membranes (17). If the inability to form colonies at concentrations of AMB above 0.5 μg/ml was due solely to

cell death, it is difficult to understand how these cells were able to maintain some degree of integrity and negative membrane potential at concentrations of amphotericin B from 0.5 to 4 μg/mL (Fig. 6.1B).

In summary, these findings indicated that concentrations of AMB between 0 and 0.4 μg/ml injured *C. albicans* and resulted in a 90% loss of their reproductive capacity, complete reduction of intracellular esterase activity (CFDA) and a reduction of intracellular ATP by almost one-hundred fold. The mortality-specific dyes showed that despite sustaining this degree of injury that a negative membrane potential and cellular integrity continued to be maintained. Only at concentrations higher than 1 μg/ml of AMB did cell death begin to occur as evidenced by $DiBAC_4(3)$ and SYBR Green I staining. At concentrations higher than 4 μg/ml of AMB, there was no further increase in mortality-specific dye fluorescence. These findings confirmed the results we reported previously (22).

We have conducted further studies on the behavior of *C. albicans* exposed to concentrations of AMB greater than 0.4 μg/ml. The observed decrease in intracellular ATP (Fig. 6.1C) prompted the investigation of an additional parameter to monitor the production of ATP. The tetrazolium salt XTT has been used to measure the activity of the succinate-tetrazolium reductase system, which is part of the

respiratory chain in mitochondria and is involved in the production of ATP (31, 34). Fig. 6.1D shows that XTT reduction increased as *C. albicans* was exposed to AMB concentrations from 0.2 μg/ml rising to a peak at 0.5 μg/ml and then declining to a minimum at concentrations above 0.8 μg/ml (Fig. 6.1D). To date, this is the only metabolic marker we have found that indicates activity in cells exposed to this concentration range of AMB (Fig. 6.4A). We postulate that the reduction of XTT is a marker for a state in which the yeast were incapable of reproduction but were not yet fatally injured. It has been previously suggested that intact cells that no longer have the ability to form colonies may retain some metabolic activity and that different assays used to determine viability are measuring different fractions of the total population (19).

As these nonreplicating *C. albicans* cells were sublethally injured the cells may have the capacity to recover from injury and undergo a restoration of replication (7). Consequently, we investigated conditions that would restore the capacity of *C. albicans* to replicate on agar plates. When *C. albicans* treated with AMB for 10 hr at 35°C was further incubated for 15 hr at 22°C it recovered the ability to replicate on agar that was lost during the initial 10 hr exposure at 35°C (Fig. 6.1A). The conditions used for resuscitation of replication competency were

selected based on the several lines of reasoning. First, it has been empirically shown that microorganisms isolated from the environment do not form colony forming units on solid agar medium very well, but can be induced to do so when the incubation temperature is decreased. A lower temperature may be critical for certain biosynthetic functions to occur that are required for the recovery process. Second, we were initially concerned that it would be difficult to distinguish between the outgrowth of a small surviving population of cells from the actual recovery of the ability to replicate by the entire population of cells. The suboptimal incubation temperature was surmised to be a good condition to inhibit this outgrowth, assuming that it would permit resuscitation of replication.

We surmised that one way to quickly distinguish outgrowth from resuscitation would be to compare the colony forming units and the particle counts. The particle counts should remain the same if resuscitation occurs because the cells are not lysed after exposure to amphotericin B and should not increase in number above that at time zero unless outgrowth occurs or the cells recover completely. A culture of cells that had incompletely recovered the capacity to replicate on agar can be invisioned to have a constant particle count and increasing colony forming units. This increase in colony forming units would

continue until reaching the point of complete recovery at which time the particle count and colony forming units would then be equal at time zero and continue to increase at the same value.

At a concentration of 0.7 μg/ml AMB the number of organisms capable of forming colonies increased 40 fold from $1x10^4$ to $6x10^6$ (Fig. 6.1A, Fig. 6.2A). The vitality specific dye fluorescence and intracellular ATP levels were not significantly affected (Fig. 6.1B, Fig. 6.1C). The mortality specific dyes showed a slight increase in fluorescence indicating that some cell death occurred (Fig. 6.1B). With the restoration of reproduction, the cells also showed a decrease in XTT reduction (Fig. 6.1D). The restoration of reproduction was further evidence that the *C. albicans* cells were injured and not dead at AMB concentrations above 0.4 μg/ml.

Subsequently, we investigated additional antifungal agents that could interfere with this resuscitation process and reduce the concentration of AMB required to fatally injure *C. albicans*. Miconazole was selected as a candidate because, in addition to interfering with ergosterol synthesis by inhibiting sterol C14-demethylation similar to other azole antifungal agents (32), it also has unique fungicidal activities. Fungistatic concentrations of 0.05 μg/mL of miconazole induce minimal morphologic changes at the cell periphery (13) but inhibit new hyphal outgrowth from

parent yeast cells (25). 0.5 μg/mL of miconazole has also been shown to inhibit the yeast mitochondrial ATPase (26) and increase cell volume and the number of peroxisomes (13). 5 μg/mL of miconazole significantly reduces CFUs (4) and inhibits the ATPase (35) and cytochrome oxidase (12) enzymes in the plasma membrane. 10 μg/ml of miconazole has also been shown to cause a 99% decrease in the intracellular ATP concentrations. Direct membrane damage and cellular necrosis has been shown to result with exposure to 50 μg/mL of miconazole likely due to peroxide accumulation (12, 32). For these reasons we tested *C. albicans* MY1417 during the resuscitation conditions in the presence or absence of 20 μg/ml of miconazole.

This concentration of miconazole reduced the number of cells capable of forming colonies at 0.7 μg/ml of AMB by 277 fold from $6x10^5$ to $2.1x10^3$ (Fig. 6.2A). In other words the recovery of reproductive capacity was prevented by miconazole. Furthermore, the presence of miconazole greatly decreased the concentration of AMB required to increase the fluorescence of the mortality-specific dyes $DiBAC_4(3)$ and SYBR Green I (Fig. 6.2B and Fig. 6.4). $DiBAC_4(3)$ and SYBR Green I fluorescence became nearly maximal at 0.9 μg/ml of AMB. Miconazole is known to inhibit the synthesis of ergosterol and produce direct damage to the cytoplasmic membrane (4). The $DiBAC_4(3)$ and SYBR

Green I results were likely a consequence of this. The reduction of XTT was slightly decreased (Fig. 6.2D) and the concentration of intracellular ATP was unchanged (Fig. 6.2C).

A comparison of the relative abilities of miconazole, ketoconazole, fluconazole, itraconazole, and 5-flucytosine to interfere with resuscitation at 22°C for 15 hr and thereby reduce the concentration of AMB required to fatally injure *C. albicans* was conducted by measuring the $DiBAC_4(3)$ and SYBR Green I fluorescence of the cells initially exposed to 0.5 μg/ml of AMB for 10 hr and incubated with the respective antifungal agent using resuscitation conditions. The fluorescence intensities were compared to the maximum that was measured under the same conditions in the presence of 4 μg/ml of AMB and expressed as a per cent. Simultaneously, the CFUs remaining after the sequential exposure to the two antifungal agents was measured. The results shown in Table 6.1 indicate that 50 μg/ml of fluconazole, and 10 and 100 μg/ml of 5-flucytosine did not inhibit resuscitation or increase mortality-specific staining. Of the remaining azoles tested, miconazole was the most effective at both increasing mortality specific staining and preventing resuscitation, while ketoconazole was more effective than itraconazole but less effective than miconazole. Retreatment with the same concentration of amphotericin B did not increase mortality-specific

staining above the low level that was observed when no agent was present (Fig. 6.1B and 6.3).

In summary, we have shown that an AMB concentration of 0.4 μg/mL was sufficient to reduce CFUs by 90%. This reduction was unlikely to be due to the death of the organisms because they: (i) continued to demonstrate XTT reduction, (ii) were capable of restoring reproduction after incubation at 22°C for 15 hr in the continued presence of AMB, and (iii) could be further damaged by adding miconazole.

Previous users of the conventional fungicidal assay have interpreted the failure of amphotericin-treated *C. albicans* to form colonies after plating on nutrient agar as meaning that the cells were dead. Other explanations are possible. Firstly, amphotericin causes a lengthy postantifungal effect that inhibits replication with a duration between 5 and 10 hr for *C. albicans* depending on the concentration of AMB and time of exposure (15, 30, 33).

The postantifungal effect is a suppression of fungal growth that persists after limited exposure to an antifungal agent (33) and may have therapeutic relevance in determining the antifungal dosing regimes (11). Empirical observations have shown that the lower temperatures decrease the duration of the postantifungal effect (16) and also may reduce the length of recovery time for injured fungal cells (20).

Sublethally stressed fungal cells exhibit a lag before the onset of replicative growth due to the repair of cell damage (9) and it has been suggested that the duration of the postantifungal effect induced by AMB may be related to the time required for yeast cell wall damage to be repaired (33). Secondly, AMB may not have been effectively removed by dilution when plating (29). AMB is more likely to remain bound to the cell membrane of *C. albicans* after plating than to diffuse into the plate medium buffer. In the azoles it is well established that their postantifungal effect and lipophilia are correlated (29), and the nonlipophilic fluconazole is the only one to lack a postantifungal effect (15, 29).

If AMB concentrations previously interpreted as cidal are in fact injuring yeast so that they cannot reproduce and this defect is reversible, then clinical failures encountered with this antifungal agent are more understandable. There are also other clinically confounding reasons, such as differences in the conditions at the site of infection, which may explain why some patients fail treatment (28). Different tissues do not receive the same exposure to the same concentrations of amphotericin B. Also *C. albicans* may be protected under some circumstances such as in focal lesions in the liver and spleen or heart valve vegetations. The clinical treatment of *C. albicans* with conventional intravenous

doses of AMB achieves peak serum levels between 0.5 to 2 μg/mL that fall rapidly and then slowly reach a plateau between 0.2 and 0.5 μg/mL (6). The risk of permanent renal impairment and the difficulties with solubility of this agent prevent the use of dosing that would achieve 4 μg/mL of AMB (8).

Our findings indicate that *C. albicans* AMB sustains concentration dependent injuries that can differentially compromise key cell functions. At concentrations of AMB between 0 and 0.5 μg/mL, intracellular esterase activity, intracellular ATP, and reproduction are greatly reduced. At intermediate concentrations of AMB between 0.5 and 4 μg/mL, cell membrane potential and integrity are affected and mitochondrial ATP pathways show an increase in activity based on XTT reduction (Fig. 6.4A). Above 4 μg/mL all activity ceases. At concentrations lower than 4 μg/mL, the ability to reproduce is recoverable after 15 hr at 22°C. Thus, the inability of the cell to reproduce in a fungicidal assay does not represent cell death. The discrepancy may be due to the postantifungal effect, hydrophobicity of the drug, inability of the yeast to undergo repair in the time available or some combination of these. It may be necessary to interpret conventional assays in the future more carefully by confirming with mortality-specific dyes if one of these conditions is present.

The clinical relevance of the incubation temperature of 22°C, that resulted in the recovery from injury, is not explicity obvious because it is lower than the temperature of the human host. However, by showing that *C. albicans* was not killed *in vitro* with concentrations of amphotericin B that are normally achieved *in vivo*, this suggests that outgrowth *in vivo* may be possible. This outgrowth of *C. albicans in vivo* could result from the same or related cellular mechanisms that are initiated with incubation at 22°C.

AMB has been a mainstay of therapy against systemic fungal disease since its introduction. Throughout that time, failures after seemingly adequate drug levels have been achieved have been clinically disappointing with serious consequences for patients. AMB may not be able to achieve sufficient prolonged concentrations in some cases to effect lethal damage. Our results indicate that the fungal cells are only sublethally injured at concentrations of AMB that are interpreted conventionally as fungicidal. Combination therapy with agents or combinations of agents that show significant mortality-specific dye activity in therapeutic ranges merit further investigation.

IV. TABLES

TABLE 6.1. Summary of the plate counts and mortality-specific staining for the treatment of *C. albicans* MY2417 with amphotericin B for 10 hr at 35°C followed sequentially for 15 hr at 22°C with the addition of a 2nd antifungal agent

Antifungal Agent	% of maximum fluorescence		CFU/mL
	$DiBAC_4(3)$	SYBR Green I	
growth control	3.2	5.5	$1.1x10^7$
50 μg/mL fluconazole	3.6	8.7	$1.0x10^7$
10 μg/mL 5-flucytosine	2.7	5.5	$6.6x10^6$
100 μg/mL 5-flucytosine	3.4	5.8	$3.6x10^6$
50 μg/mL itraconazole	17.9	31.5	$2.8x10^6$
50 μg/mL ketoconazole	36.5	58.5	$8.5x10^5$
10 μg/mL miconazole	81.5	31.1	$9.4x10^3$
20 μg/mL miconazole	76.1	63.6	$8.5x10^3$

V. FIGURES

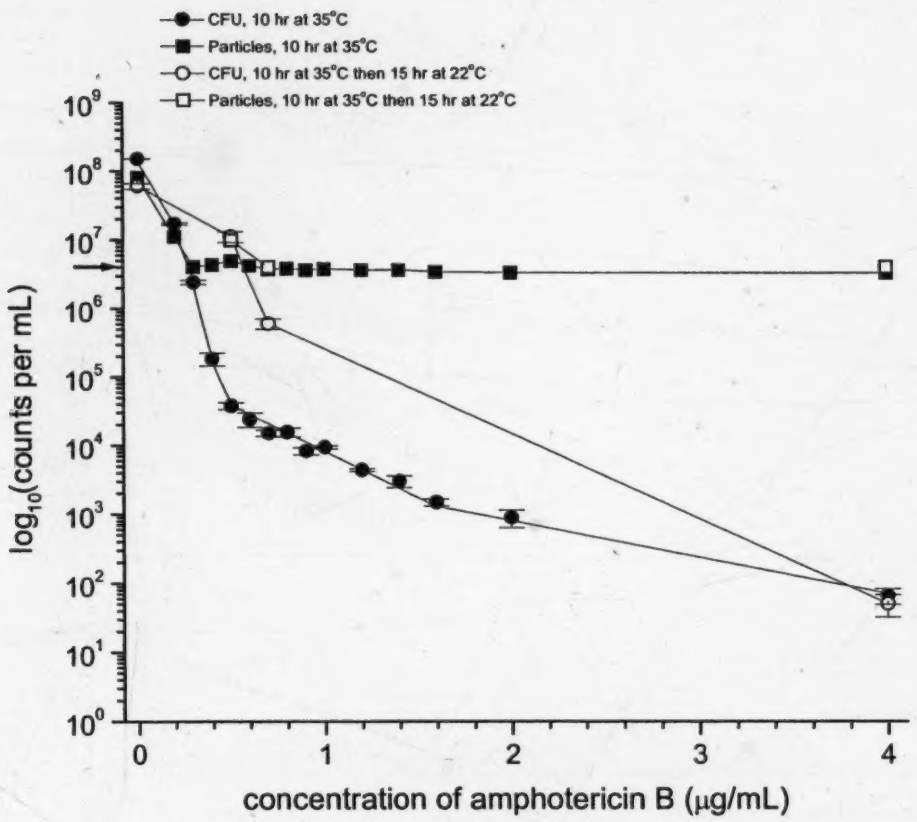

CFU, 10 hr at 35°C
Particles, 10 hr at 35°C
CFU, 10 hr at 35°C then 15 hr at 22°C
Particles, 10 hr at 35°C then 15 hr at 22°C
log10(counts per mL)
concentration of amphotericin B (μg/mL)

Fig. 6.1A. Plate counts (CFU/mL) and particle counts per mL for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C followed by 15 hr at 22°C. ●, CFU/mL for 10 hr at 35°C; ■, particle counts/mL for 10 hr at 35°C; ○, CFU/mL for 10 hr at 35°C followed by 15 hr at 22°C; □, particle counts/mL for 10 hr at 35°C followed by 15 hr at 22°C; , CFU/mL and particle counts/mL at time zero. Error bars indicate standard error. →, measurements taken at time zero.

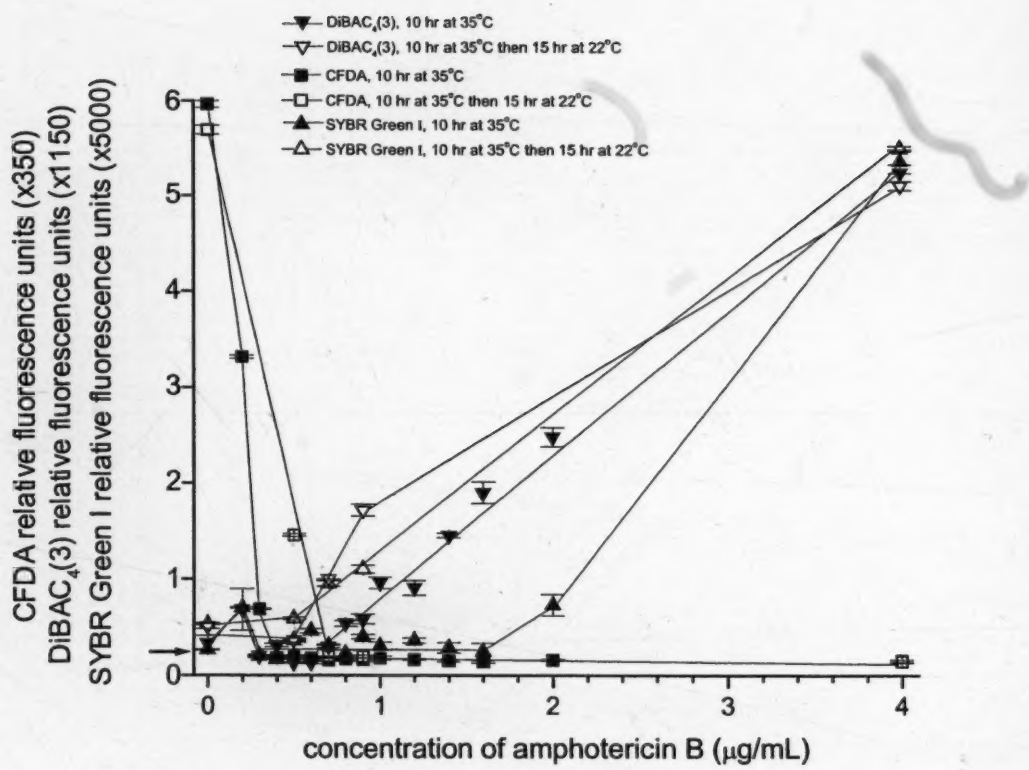

DiBAC4(3), 10 hr at 35°C
DiBAC4(3), 10 hr at 35°C then 15 hr at 22°C
CFDA, 10 hr at 35°C
CFDA, 10 hr at 35°C then 15 hr at 22°C
SYBR Green I, 10 hr at 35°C
SYBR Green I, 10 hr at 35°C then 15 hr at 22°C
CFDA relative fluorescence units (x350)
DiBAC4(3) relative fluorescence units (x1150)
SYBR Green I relative fluorescence units (x5000)
0
1
2
3
4
5
6
concentration of amphotericin B (μg/mL)

Fig. 6.1B. $DiBAC_4(3)$, SYBR Green I, and CFDA staining for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C followed by 15 hr at 22°C. ▼,▽, $DiBAC_4(3)$; ▲,△, SYBR Green I; ■,□, CFDA; closed,10 hr at 35°C; open, 10 hr at 35°C followed by 15 hr at 22°C; , fluorescent staining values at time zero. Error bars indicate standard error. →, measurements taken at time zero.

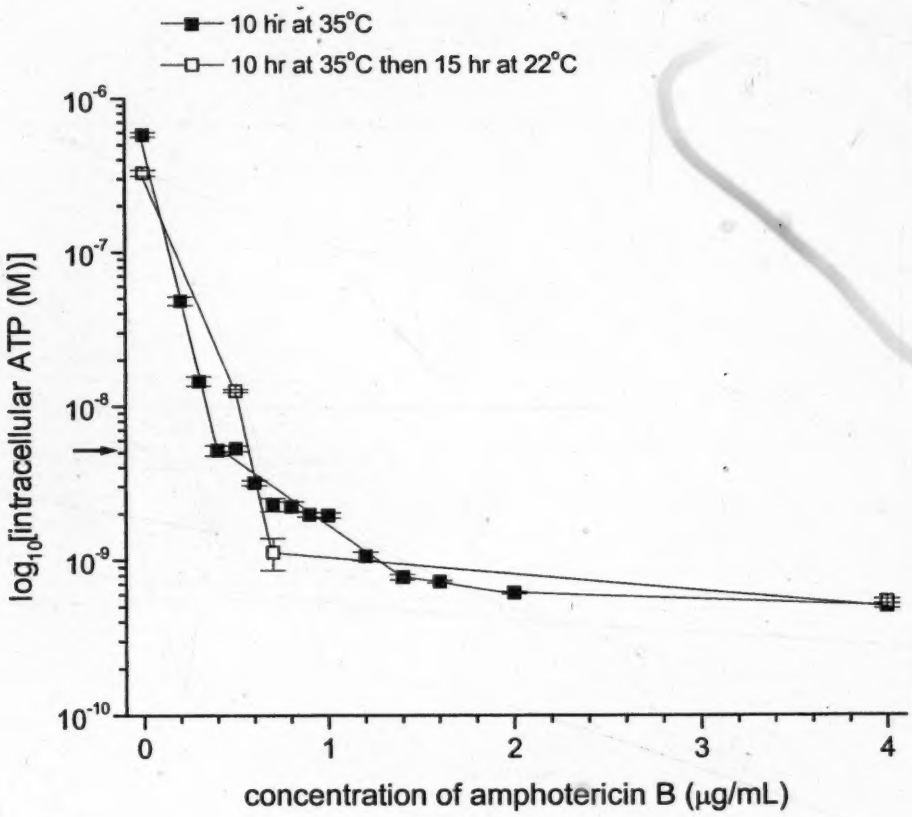


Fig. 6.1C. Intracellular ATP concentration (M) for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C and 10 hr at 35°C followed by 15 hr at 22°C. ■, 10 hr at 35°C; □, 10 hr at 35°C followed by 15 hr at 22°C; , intracellular ATP concentration at time zero. Error bars indicate standard error. →, measurements taken at time zero.

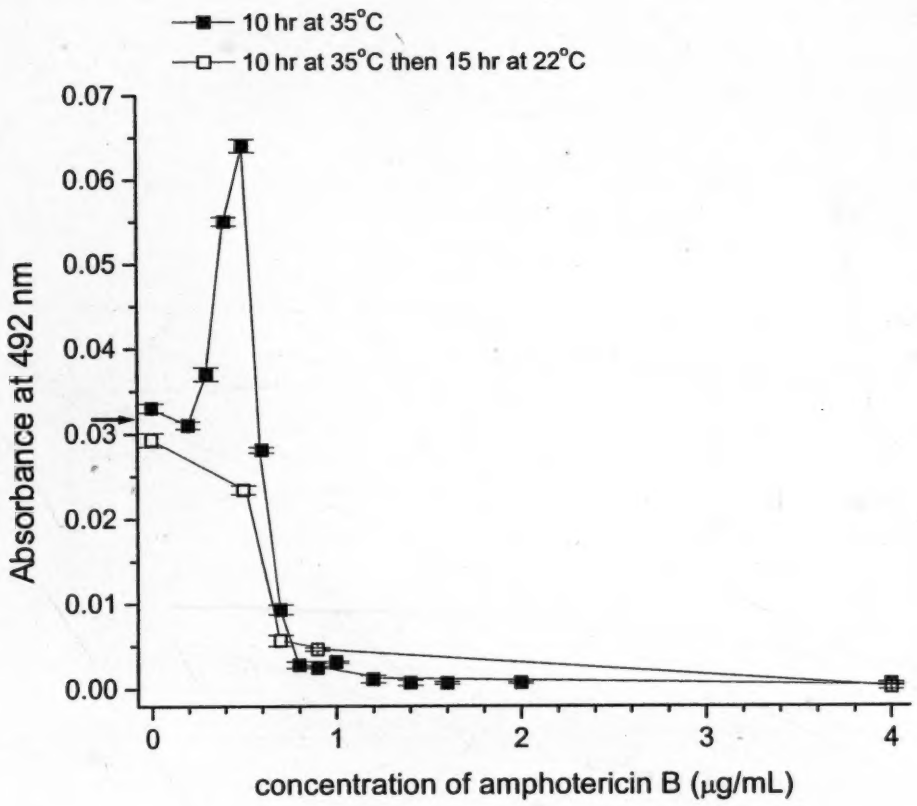


Fig. 6.1D. XTT reduction for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C and 10 hr at 35°C followed by 15 hr at 22°C. ■, 10 hr at 35°C; □, 10 hr at 35°C followed by 15 hr at 22°C; , XTT reduction at time zero. Error bars indicate standard error. →, measurements taken at time zero.

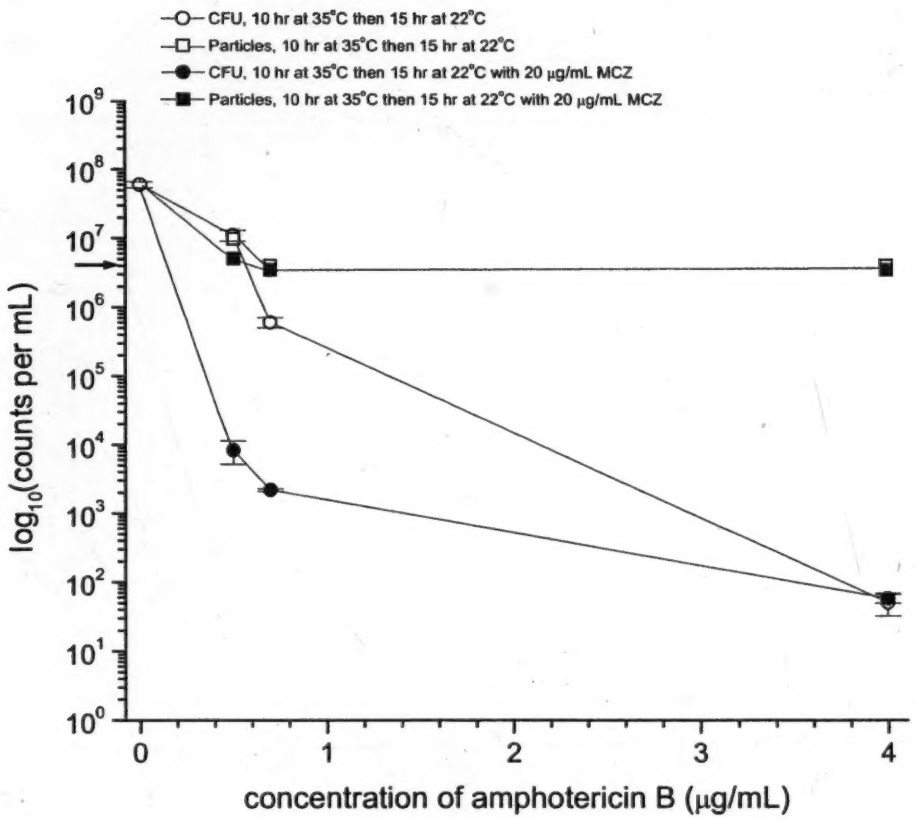
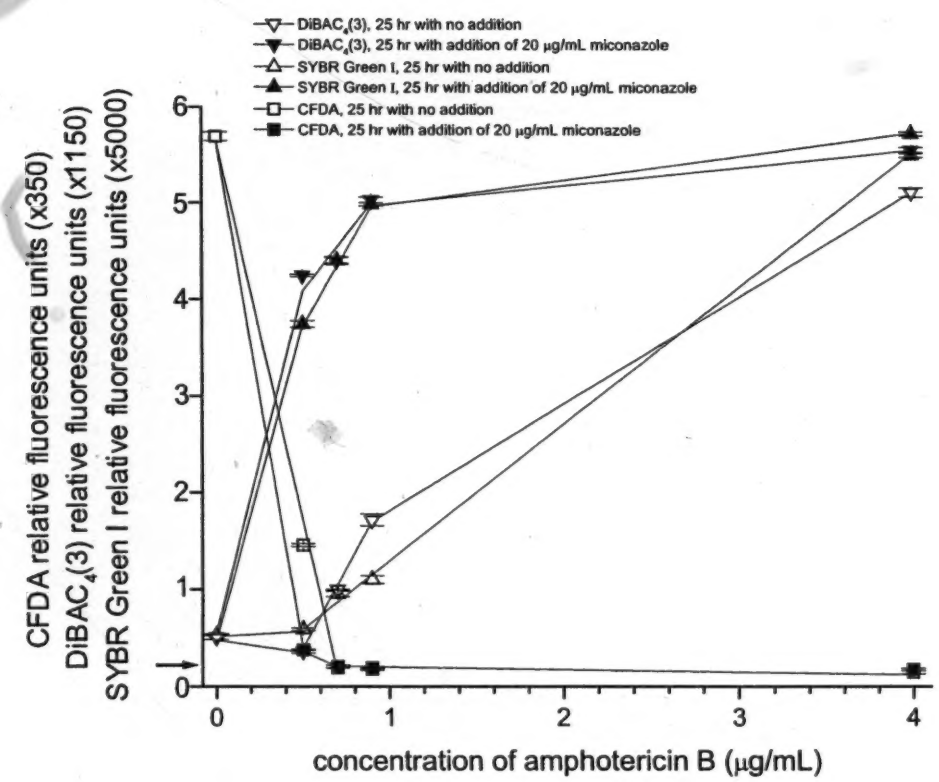

CFU, 10 hr at 35°C then 15 hr at 22°C
Particles, 10 hr at 35°C then 15 hr at 22°C
CFU, 10 hr at 35°C then 15 hr at 22°C with 20 μg/mL MCZ
Particles, 10 hr at 35°C then 15 hr at 22°C with 20 μg/mL MCZ
log10(counts per mL)
concentration of amphotericin B (μg/mL)

Fig. 6.2A. Plate counts (CFU/mL) and particle counts per mL for *C. albicans* strain MY2417 incubated with amphotericin B for 10 hr at 35°C followed by 15 hr at 22°C or 20 μg/mL of miconazole for 15 hr at 22°C. ○, CFU/mL for 10 hr at 35°C followed by 15 hr at 22°C; □, particle counts/mL for 10 hr at 35°C followed by 15 hr at 22°C; ●, CFU/mL for 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL of miconazole; ■, particle counts/mL for 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL of miconazole. , CFU/mL and particle counts/mL at time zero. Error bars indicate standard error. →, measurements taken at time zero.


DiBAC4(3), 25 hr with no addition
DiBAC4(3), 25 hr with addition of 20 μg/mL miconazole
SYBR Green I, 25 hr with no addition
SYBR Green I, 25 hr with addition of 20 μg/mL miconazole
CFDA, 25 hr with no addition
CFDA, 25 hr with addition of 20 μg/mL miconazole
CFDA relative fluorescence units (x350)
DiBAC4(3) relative fluorescence units (x1150)
SYBR Green I relative fluorescence units (x5000)
0
1
2
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6
concentration of amphotericin B (μg/mL)

Fig. 6.2B. $DiBAC_4(3)$, SYBR Green I, and CFDA staining for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C followed by 15 hr at 22°C or 20 μg/mL of miconazole for 15 hr at 22°C. ▼,▽, $DiBAC_4(3)$; ▲,△, SYBR Green I; ■,□, CFDA; closed, 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL of miconazole; open, 10 hr at 35°C followed by 15 hr at 22°C. , fluorescent staining values at time zero. Error bars indicate standard error. →, measurements taken at time zero.

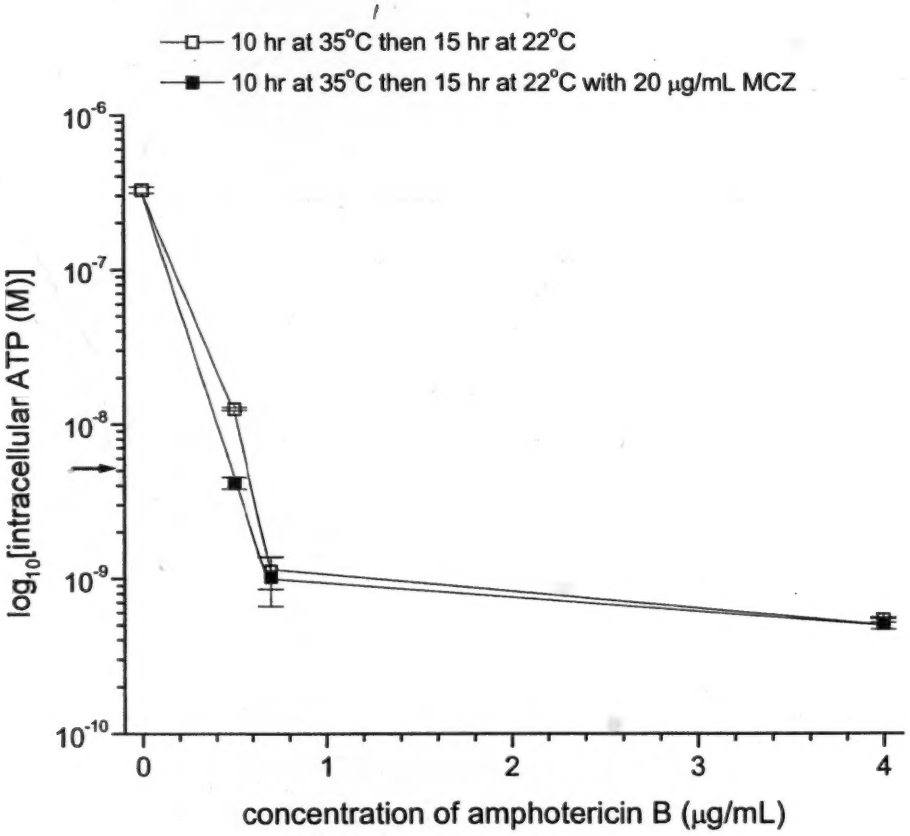


Fig. 6.2C. Intracellular ATP concentration (M) for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C followed by 15 hr at 22°C or 20 μg/mL of MCZ for 15 hr at 22°C. □, 10 hr at 35°C followed by 15 hr at 22°C; ■, 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL of MCZ. , intracellular ATP concentration at time zero. Error bars indicate standard error. →, measurements taken at time zero.

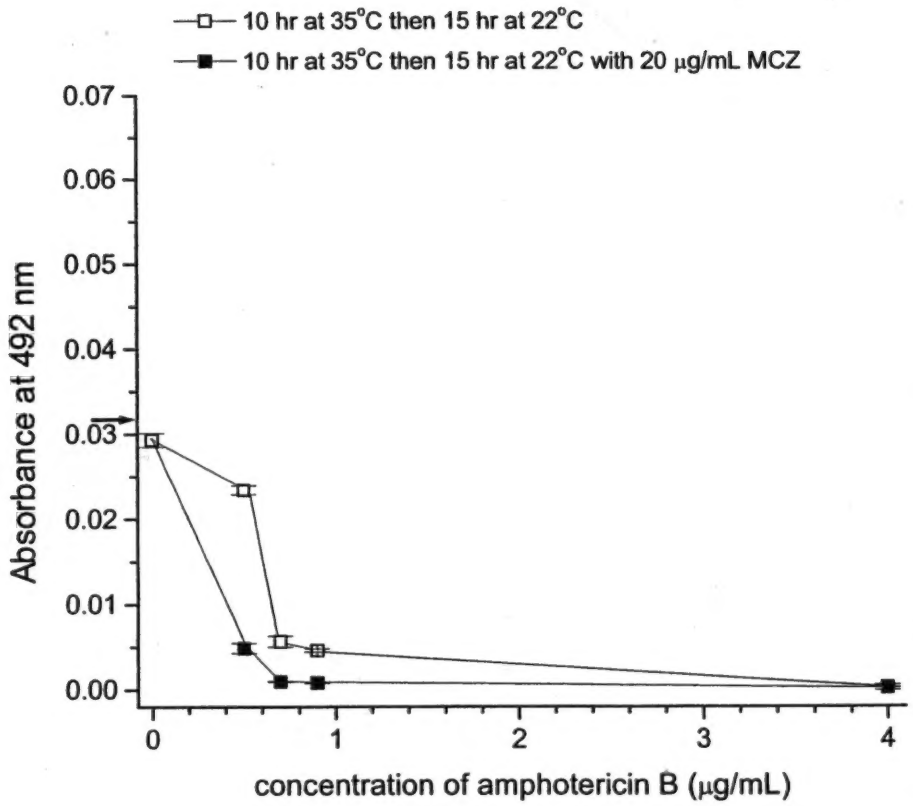


Fig. 6.2D. XTT reduction for *C. albicans* strain MY2417 incubated with AMB for 10 hr at 35°C followed by 15 hr at 22°C or 20 μg/mL of MCZ for 15 hr at 22°C. □, 10 hr at 35°C followed by 15 hr at 22°C; ■, 10 hr at 35°C followed by 15 hr at 22°C with 20 μg/mL of MCZ. , XTT reduction at time zero. Error bars indicate standard error. →, measurements taken at time zero.

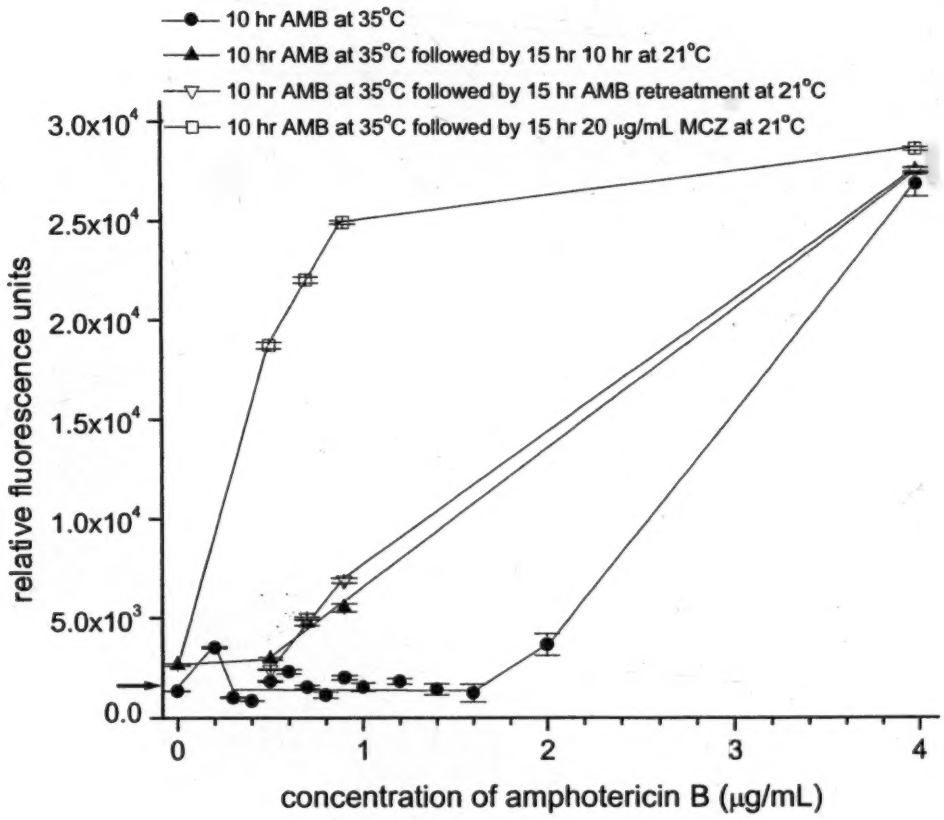
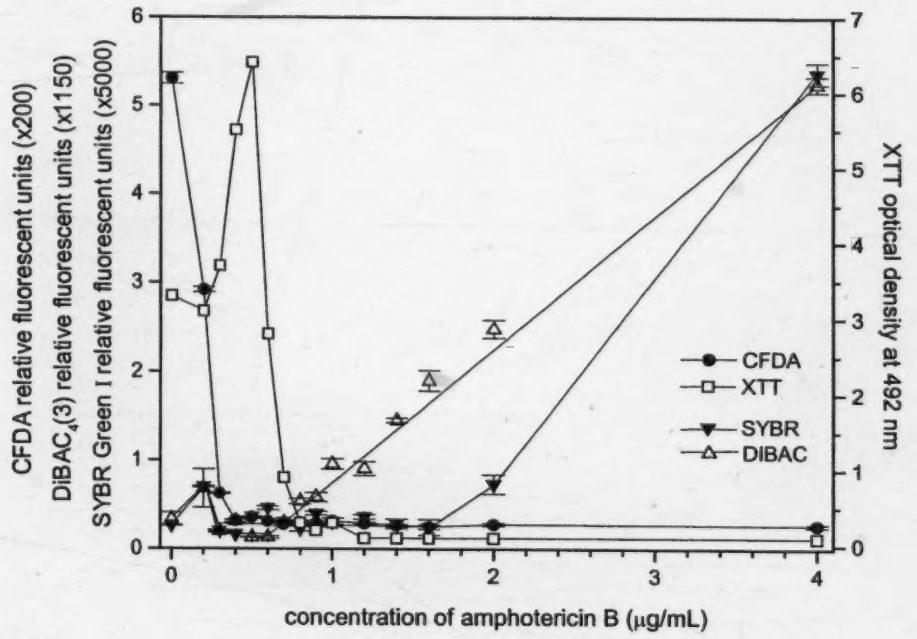


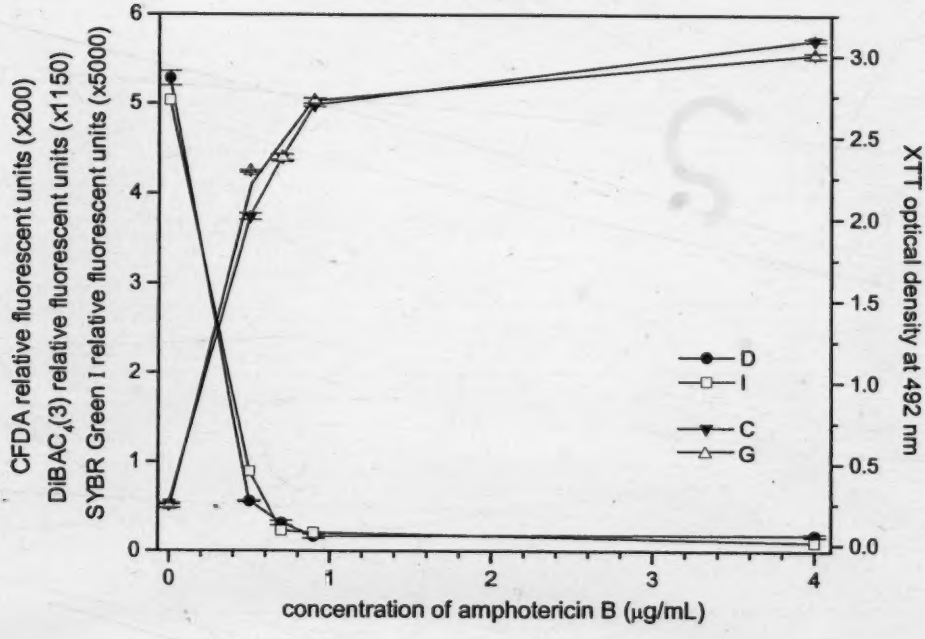
Fig. 6.3. SYBR Green I staining. Time-kill curve for *C. albicans* strain MY2417 incubated first with amphotericin B (AMB) for 10 hours at 35°C. The amphotericin B-exposed cultures were subsequently incubated for 15 hours at 22°C under 3 different conditions: (i) without the addition of a new agent allowing for recovery, (ii) retreatment with the same concentration of amphotericin B that the cultures were exposed to over the first 10 hr, and (iii) retreatment with 20 μg/mL of miconazole (MCZ). Error bars indicate standard error.→, measurements taken at time zero.

Fig. 6.4. Time-kill curves for *C. albicans* strain MY2417 incubated with amphotericin B for 10 hours at 35°C (A). Sequential time-kill curves showing the treatment of amphotericin B-exposed cultures with 20 μg/mL of miconazole for 15 hours at 21°C (B). The time-kill curves are both evaluated using vitality-specific parameters (CFDA staining and XTT reduction) and mortality-specific parameters ($DiBAC_4(3)$ staining and SYBR Green I staining). Error bars indicate standard error. The XTT y-axis scale in (A) is reduced in (B).

A.


CFDA relative fluorescent units (x200)
DiBAC4(3) relative fluorescent units (x1150)
SYBR Green I relative fluorescent units (x5000)
XTT optical density at 492 nm
CFDA
XTT
SYBR
DIBAC
concentration of amphotericin B (μg/mL)

B.


CFDA relative fluorescent units (x200)
DiBAC4(3) relative fluorescent units (x1150)
SYBR Green I relative fluorescent units (x5000)
XTT optical density at 492 nm
D
I
C
G
concentration of amphotericin B (μg/mL)

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Chapter 7

DISCUSSION

I. Summary

The simultaneous measurements of qualitative and quantitative characteristics of cells within a suspension can be achieved with great precision with fluorescent dyes that have specific cellular affinities (63). We used a combination of vitality-specific and mortality-specific fluorescent dyes to assess overall yeast cell viability. The definition of vitality being used here was put forward by Lentini and is a function of the total yeast cell viability as determined by agar plate counts and the physiological state of that population (40). Thus, vitality was evaluated by taking into account both the enumeration of colony-forming units and the staining with vitality-specific dyes, intracellular ATP concentration, and the reduction of XTT to formazan. Some fluorescent dyes are accumulated depending on membrane potential, which is a property of live cells. 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$) is a lipophilic, cationic dye that accumulates inside negatively charged cell compartments. If the cell membrane is damaged then its electrical potential collapses and the fluorescent dye is lost from the depolarized cell (59). A second vitality dye used was CFDA. CFDA is a lipophilic, nonpolar, fluorescein conjugate that is cleaved by non-specific, intracellular esterases to release the fluorescent, polar carboxyfluorescein, which stains the cell

(66). Dead cells do not possess the active enzymes required for cleaving of the fluorescent conjugate or lack intact membranes, allowing the fluorescein to leak out.

The direct application of CFDA and ATP was shown to benefit susceptibility testing of *C. albicans*. Endpoint determination when testing azole antifungal agents continues to be problematic, subjective, and a major source of interlaboratory variability (18). The trailing-growth phenomenon is well understood to be the singular greatest cause of these difficulties in clinical laboratories (17, 24, 69). With the incorporation of CFDA into the microdilution broth susceptibility testing format, we were able to completely eliminate this problem when testing with fluconazole (46). The trailing-growth phenomenon can also be exacerbated over time for some strains of *C. albicans* producing the low-high phenotype. These strains are highly problematic to the clinical mycology laboratory as they are sensitive at 24 hr but resistant at 48 hr when they are measured. The CFDA-modified microdilution method showed that these strains were in fact susceptible (46) confirming *in vivo* work done by Rex *et al.* using a murine model of invasive candidiasis (68). The CFDA-modified microdilution method was further confirmed for *C. tropicalis, C. glabrata,* and *C. parapsilosis* (46) using fluconazole

and with *C. albicans* using amphotericin B, ketoconazole, itraconazole, voriconazole, and flucytosine (47).

Using of intracellular ATP as an indicator of metabolic activity was also evaluated with fluconazole and amphotericin B susceptibility testing (49). This work confirmed the previous findings with the low-high phenotype strains of *C. albicans* and fluconazole using a broth macrodilution susceptibility testing method. Additionally, using this same format with amphotericin B, much shorter endpoints of 30 min and 3 hr could be obtained compared to the recommended 48 h (56).

The difficulties in quantifying microbial killing are due in large part to the properties that are attributed to the state of being alive, since the presence of dead microbes must be inferred retrospectively from the estimates of these properties (45). Microbial cell death has been traditionally defined as the irreversible loss of the ability to reproduce with the empirical test being the ability to produce colony-forming units on agar (26). However, from the outset we considered this definition to be insufficient and considered methods to directly assess mortality (Fig. 7.1). We used fluorescent dyes to do this and referred to them as mortality-specific dyes based on their cellular affinities. SYBR Green I is a fluorescent dye that is excluded by intact cells. SYBR Green I enters damaged cells with reduced plasma membrane integrity and intercalates

into double stranded DNA (23). The other dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] is a lipophilic, anionic dye that penetrates damaged cells that can not maintain a negative internal charge and binds to lipid-rich intracellular components (13).

The fungicidal activity of amphotericin B permitted the use of the mortality-specific dyes along with the vitality-specific measurements to assess the effect of amphotericin B on *C. albicans* using a kill-curve assay. We were able to delineate several stages produced as a consequence of the incubation of *C. albicans* with amphotericin B for 10 hr that included at its simplest: alive, sublethally injured and lethally injured (45). Additional work showed that the sublethally injured *C. albicans* that were previously characterized only by an absence of vitality-specific and mortality-specific staining were in fact highly metabolically active by XTT reduction. Furthermore, these sublethally injured cells could be both resuscitated with additional incubation and be lethally injured with sequential treatment with miconazole (48).

II. Sublethal Injury and Resuscitation

If one considers the traditional measurement of the outcome of an antifungal susceptibility test, a consideration of the organisms that can not replicate and produce colony-forming units but are recoverable after appropriate resuscitation is not included. The simplest reasoning for this exclusion is the absence of any standardized methods available to the clinical laboratory to take this last category into account and also a poor understanding of the existence and potential importance of this physiological state. The measurement of fungicidal killing, based on the inability to reproduce, is both misleading and an overestimate as it is well accepted that only a fraction of stressed yeast can be enumerated using the agar plate method (52). The process of cell replication deactivation is believed to involve stepwise changes in the physiological state of a cell that render an intermediate form incapable of initiating replicative processes but still capable of metabolism (28, 65). Thus the loss of replication competency is an early event in a phase of decline that leads to eventual cell death (65) (Fig 7.1).

Our vitality-specific and mortality-specific assays were able to describe for the first time amphotericin B-treated *C.albicans* that belong to this category of nonreplicating, sublethally injured cells. The *C. albicans*

cells exposed to between 0.4 and 1.0 µg/mL of amphotericin B for 10 hr at 35°C had a >99% inhibition of agar plate counts, did not show uptake of vitality- or mortality-specific dyes, but had very high XTT reduction (45, 48). A convenient shorthand used to refer to cells in this physiological state was 'sublethally injured'. The tetrazolium salt XTT is cleaved to formazan by the succinate-tetrazolium reductase system that belongs to the respiratory chain of the mitochondria and is active only in viable cells (74). Many oxidative enzymes, including succinate dehydrogenase, have been described for *C. albicans* (55, 84). Therefore, the amount of formazan dye formed by the mitochondrial dehydrogenase enzymes directly correlates to the number of metabolically active cells in the culture (72). The XTT assay showed that sublethally injured *C. albicans* had metabolic activity that was 50% higher than that present in the growth control (48).

One obvious explanation for the greatly increased metabolic activity in the sublethally injured cells was that they were undergoing a repair process in response to injury. It is recognized that exposure to sublethal stresses may result in the loss of the capability to initiate replicative processes under conditions that are satisfactory for unstressed cells (28, 73). Additionally, it has been suggested that sublethally injured yeast cells may retain metabolic capacity, and perhaps even at a greater rate

than that of a cell capable of replication (28). Energy generation can still occur at a high rate even though replication has stopped (27). The amount of XTT formazan produced after the initial amphotericin B exposure was proportional to the extent of the recovery of replication competency following resuscitation.

Cells are classified as injured rather than dead when they have the capability to regain a normal physiological state concomitant with the initiation of growth and cell division (73, 25). Restoring of the capacity to grow on solid medium occurs before normal growth can return (12). The sublethally injured *C. albicans* cells treated with 0.5 μg/mL of amphotericin B for 10 hr at 35°C were resuscitated with an additional incubation for 15 hours at 22°C without the removal of amphotericin B (Fig. 6.1A). Replication competency completely recovered from the initial decrease at time zero and additional growth was evaluated by the concomitant increase in the plate counts and particle counts. These resuscitated cells also converted from not staining with vitality-specific dyes at 10 hr to staining with CFDA after the 15 hr incubation required for resuscitation. In contrast, *C. albicans* treated with 0.7 μg/mL of amphotericin B for 10 hr at 35°C followed by incubation at 22°C for 15 hr without removal of amphotericin B resulted in an incomplete recovery of replication competency (Fig. 6.1A). It remains to be determined whether

continued incubation at 21°C beyond 15 hr would have resulted in continued recovery of replication competency followed by growth.

The length of time that *C. albicans* can exist in this injured, nonreplicating state before losing the capacity to undergo resuscitation is also not known. It is easy to conceptualize how a reduction in the enumeration of *C. albicans* colony-forming units from a patient with invasive candidiasis might be construed as though the patient were responding to therapeutic doses of amphotericin B, possibly leading to the conclusion that the infection was being resolved. In reality the cells might only be sublethally injured. Given that many of these patients are also immunosuppressed, it is interesting to speculate what effect the physiological environment would have on these sublethally injured *C. albicans*.

III. Sequential Combination Therapy

The use of inhibitors during the repair process can provide information on the nature of the repair mechanism. A number of antifungal agents were used in this way for sequential treatment of the sublethally injured *C. albicans* using the same resuscitation conditions. Microorganisms

that have been damaged but not killed by exposure to differing chemical or physical processes are frequently more sensitive to other types of agents (73). A number of well-established mechanisms of antimicrobial interaction exploit this to produce synergistic inhibition (16). A common strategy for selected bacterial infections is combination therapy with agents that exhibit either an additive or synergistic effect when given simultaneously. There are several potential advantages to combination antifungal therapy that include achieving a more rapid antifungal effect and allowing reduction in the dosage, and hence toxicity, of individual agents such as amphotericin B (43). The selection of antifungal combination therapy has been slowed by nonstandardized *in vitro* susceptibility testing methods and the lack of controlled clinical trials (42, 43). Moreover, considerable debate in mycology exists over what constitutes synergy or antagonism *in vitro* and whether these results are fully representative of the antifungal interaction *in vivo*.

Amphotericin B is believed to alter the cell membrane and potentiate the uptake of 5-flucytosine into the fungal cell (53). The combination of amphotericin B and 5-flucytosine was once considered standard therapy in Europe (15). This combination is effective but has not been shown to be more efficacious than fluconazole monotherapy for treating fungemia or sepsis due to *Candida* species (1). The using of amphotericin B in

combination with an azole is controversial because of the potential for antagonism based on *in vitro* data and their modes of action (22, 43, 44). It has been suggested that the azole agents may deprive amphotericin B of its binding site on the fungal cell membrane through inhibition of ergosterol synthesis (76). The timing and the order of administering the amphotericin B and fluconazole combination may greatly influence the extent and impact of this interaction (44). The concomitant use of this combination is not supported by data from current published prospective clinical trials (43), however this does not necessarily preclude their use sequentially.

A growing clinical practice has also been to give various antifungal agents in sequence for aggressive treatment of serious, invasive infections in patients who are persistently immunocompromised (14). Sequential combination therapy of antifungal agents mimics our *in vitro* time-kill curve assay used with *C. albicans*, where amphotericin B was followed with a battery of different antifungal agents to prevent the resuscitation process (48). Sublethally injured *C. albicans* produced after the treatment with concentrations of amphotericin B, normally achieved *in vivo*, between 0.5 and 0.9 μg/mL for 10 hr at 35°C, were converted to having a high intensity of mortality-specific fluorescence after the sequential treatment with 20 μg/mL of miconazole for 15 hr at

21°C (Fig. 6.4B). These observations along with the concomitant decrease in XTT reduction showed that the sequential miconazole treatment had sufficiently damaged the sublethally injured *C. albicans* to produce lethal injury and true death.

The concomitant treatment of *C. albicans* with amphotericin B and miconazole has been shown to be potentiating after prolonged exposure and antagonistic after short-term incubations (11). However, no studies investigating the sequential treatment of *Candida* with amphotericin B and miconazole exist in the literature. Pretreatment of *Aspergillus fumigatus* with amphotericin B followed by miconazole resulted in better *in vitro* effects compared with the effect of simultaneous use of the agents (51).

Primary treatment with amphotericin B followed by fluconazole or itraconazole for maintenance therapy is common with cryptococcal meningitis in patients with HIV infection (81). This regime is also commonly used clinically with *C. albicans* infections, where an initial course of amphotericin B as empiric therapy is switched to fluconazole after an invasive *C. albicans* infection is found to be susceptible or if prolonged therapy is required (4, 14, 50, 77). When studied in a rabbit model of endocarditis and pyelonephritis, the sequential use of amphotericin B for 24 h followed by fluconazole monotherapy has been

shown to have no additive effect or adverse interaction on *C. albicans* infection (50). A negative interaction has been observed with the sequential use of amphotericin for 5 days followed by itraconazole by 5 days in treating murine invasive candidiasis (77).

We also obtained comparable results *in vitro* where sequential treatment showed that fluconazole did not inhibit resuscitation or increase mortality-specific staining. Of the remaining azoles tested, miconazole was by far the most effective at both increasing mortality specific staining and preventing resuscitation, while ketoconazole was more effective than itraconazole.

The sequential treatment with 50 μg/mL of ketoconazole produced the next best inhibitory effect compared to 20 μg/mL of miconazole (Table 6.1). Both of these agents are imidazoles and have a complex mode of action, inhibiting not only 14α-demethylase, but also membrane-bound enzymes and membrane lipid biosynthesis (22). Both miconazole and ketoconazole inhibit the activities of cytochrome *c* peroxidase and NADH oxidase in the mitochondria, resulting in the net increase of hydrogen peroxide (5). However, the mitochondrial enzyme activities of succinate dehydrogenase and ATPase have been shown to be more sensitive to miconazole than to ketoconazole (71, 80). Miconazole was also shown by us to decrease XTT reduction to a greater extent than

ketoconazole. Miconazole can also selectively inhibit both the mitochondrial ATPase and at higher concentrations the plasma membrane ATPase (64). Thus mitochondrial enzymes are excellent candidates for being involved in both the suspected recovery mechanism and the killing process that converts the sublethally injured *C. albicans* to being lethally injured.

Miconazole has serious toxicity issues and is not a specific inhibitor of fungal cells. For instance, miconazole is used as an ATPase inhibitor in a number of different eukaryotic cell types. Efforts to find a more specific inhibitor with a more limited and better understood mechanism of action that could abort the resuscitation process were unsuccessful.

IV. Amphotericin B Postantifungal Effect

Very little is known about the pharmacodynamics of antifungal agents (3). Pharmacodynamics attempts to characterize the changing concentration of a drug at its site of action and its ability to affect microbial growth over time. Dosing strategies based on pharmacodynamic principles have been successfully applied in managing bacterial infections (19). Dosing of antifungal agents has

been traditionally guided by their respective pharmakokinetic properties in attempts to achieve adequate concentrations at the site of infection while minimizing treatment-related toxicity (60, 78). Complex interactions between the antifungal agent, host, and fungal pathogen are rarely characterized and are thus not considered when constructing therapeutic regimens (14).

Determining the optimum dosage of an antimicrobial agent requires knowing the *in vitro* MIC, because the dosing regimen for antifungal agents with concentration-dependent activity is designed to obtain the highest maximum concentration:MIC ratio. The National Committee for Clinical Laboratory Standards has developed guidelines to allow for the development of standard breakpoints to guide therapy and decrease interlaboratory variability (56). The clinical usefulness of *in vitro* susceptibility testing results, in treating invasive fungal infections, remain somewhat controversial and imprecise. Most pharmacodynamic studies are limited by the availability of a model that accurately reflects pharmacokinetic properties in humans (14). Given the narrow therapeutic window of amphotericin B and frequent treatment failures, it has been suggested that there is a need for a reevaluation of the current dosing regimens (3).

The postantibiotic effect concept that so greatly changed dosing regimens for aminoglycoside therapy of bacteria has been examined for its potential impact in antifungal agent dosing (58). The postantibiotic effect is observed when an antibacterial agent continues to have inhibitory activity after its removal from the bacteria. The clinical significance of postantifungal effect is still ill defined and has not been tested in treating human infection (14). Previous *in vitro* time-kill and postantifungal effect studies with amphotericin B have demonstrated concentration-dependent activity and a significant postantifungal effect against a variety of yeasts, including *C. albicans* (32, 79). The magnitude of the postantifungal effect *in vitro* was dependent upon the concentration of amphotericin B and the duration of exposure, ranging from 0.5 to 10 h (17, 79).

The best characterized *in vitro* method for evaluating antifungal pharmacodynamics is the time-kill curve analysis (30, 31), which was used by us to evaluate amphotericin B activity with *C. albicans*. We have shown that the conventional fungicidal assay that equates the loss of *C. albicans* replication competency to cell death may give incorrect results with amphotericin B (48). This was demonstrated by the recovery and growth of *C. albicans* cells that were exposed to 0.5 and 0.7 mg/mL of amphotericin B. We believe the inability of *C. albicans* to

form CFUs was due to sublethal injury and that a recovery process was present as evidenced by the high metabolic activity indicated by XTT reduction. It has been suggested that the duration of the postantifungal effect that is induced by amphotericin B may be related to the time necessary for yeast cell wall damage to be repaired and subsequent organism multiplication to resume (79). Thus, it would appear that in the case of amphotericin B, the underlying mechanism of the postantifungal effect is not likely one that can used to the advantage of dosing regimens if the exposed cells are undergoing repair rather than experiencing the ongoing inhibitory action of the antifungal agent. However, the postantifungal effect may have to be taken into account when considering dosing, given that the concentrations of amphotericin B at which *C. albicans* was shown to be able to recover from sublethal injury are within the narrow range of the concentrations achieved in the serum with conventional intravenous doses (7). This fact may help partly explain the requirement for prolonged amphotericin B treatment and the frequent clinical failure observed with this drug.

V. Amphotericin B: Fungicidal versus Fungistatic Activity

Amphotericin B has been described as having concentration-dependent fungicidal activity and the azole antifungal agents are described as exhibiting concentration-independent fungistatic activity (17). Concentration-dependent activity is used to describe agents, that when evaluated using a dose-response curve, have a large range of concentrations over which the transition between the levels of activity occur (32). Time-kill curve assays have been reported in several studies and showed that amphotericin B induces a three to four $\log_{10}$ decrease in CFUs in a time span of 2 to 4 h at concentrations higher than the MIC (32). The length of the incubation for our time-kill assays was extended to 10 hr where maximal mortality-specific staining was measured (45). Different alterations to the recommended NCCLS microdilution protocol for amphotericin B susceptibility testing have been suggested to improve the correlation between the MIC and clinical outcome, but a consensus has not been obtained (24). Variations in the correlation of *in vitro* results with clinical outcome may also be due, in part, to differences in the conditions at the site of infection.

Our observations, that *C. albicans* are not killed with amphotericin B at concentrations normally achieved *in vivo,* question the validity of the

understanding that amphotericin activity is fungicidal as it is currently being used. With this in mind, a reevaluation of the activity of amphotericin B described in the literature does lend support to this theory.

The minimum fungicidal concentration (MFC) is defined as the concentration which results in a 99.9% reduction in colony-forming units/mL (53), although it has been suggested that this criterion for MFC requires further validation (14). Amphotericin B fungicidal concentrations are usually 0.5 to 2 times the MIC in *Candida* species, which is usually between 0.25 and 1.0 μg/mL of amphotericin B (32). For bactericidal agents, the MIC and MBC are anticipated to be similar and it is accepted that for bacteriostatic agents that the MBC will be several dilutions greater than the MIC (2). This definition of static antibacterial agents best describes the activity observed with amphotericin B.

Kotler-Brajtburg *et al.* suggested that a fungistatic mechanism of inhibition could be discerned from a fungicidal level for amphotericin B (34). The yeast *Saccharomyces cerevisiae* incubated for 1 hr at 22°C with amphotericin B caused significant potassium leakage up to 0.6 μg/mL and at concentrations lower than those required for cell death as measured by plate counts (34). Potassium leakage is one of the earliest

observable changes in plasma membrane permeability and is used as an indicator of damage (35). Several other experimental polyene antifungal agents in the survey caused a concomitant decrease in potassium and CFUs.

In an *in vivo* study by Andes *et al.*, the best predictor of efficacy using the neutropenic-mouse disseminated-candidiasis model of infection was a peak serum concentration:MIC ratio of 10:1 or greater (3). Although there are a number of factors that can affect drug activity *in vivo,* these results provide further support for the argument that amphotericin B may not be fungicidal as it was previously thought.

It has been noted that the difference in sensitivity to amphotericin B between fungal and mammalian cells is small and that a small increase in phenotypic or genotypic resistance of *C. albicans* could render an infection insensitive to normal treatment (21). Although the resuscitation process described by us is more of a coping mechanism than a true resistance mechanism, it is possible that a similar effect is achieved *in vivo*. The fungistatic activity observed by us for amphotericin B should prompt us to evaluate our approach to the utilization and even susceptibility testing of this agent.

VI. Amphotericin B Mechanism of Action

The primary mode of action of amphotericin B is through binding to ergosterol in the plasma membranes of fungal cells. Amphotericin B binding is believed to result in disorganization and loss of integrity of the membrane, possibly by forming aqueous pores composed of small aggregates of amphotericin B (8). It is possible to speculate that the alteration of the fungal plasma membrane integrity is not the primary mechanism of killing. It is also understood that amphotericin B exerts oxidative damage to the cell that contributes to its activity (75). A frequently cited article by Beggs has stated that the simplest explanation for the lethal action of amphotericin B is one involving physiochemical cell membrane damage (6). This author also states that oxidative damage may play some role in the antifungal activity of amphotericin B, however the oxidative damage hypothesis (9) is difficult to accept as the primary explanation of lethal action, because of its greater complexity and accompanying unanswered questions (6).

Brajtburg *et al.* proposed that the lethality of amphotericin B might not be a consequence of changes in cell membrane permeability but rather due to amphotericin B-induced damage linked to the generation of reactive forms of oxygen (9). The evidence for the role of active oxygen

species in the fungicidal activity of amphotericin B was obtained from experiments which showed that amphotericin B injury to cells could be modulated when scavenged with extracellular catalase (75) and potentiated in the presence of ascorbic acid and other prooxidants (9). These oxidative effects occurred without altering the sublethal amphotericin B-induced potassium leakage commonly used to describe membrane damage. The only mechanism described in the literature that offers an explanation as to how these oxidative events might occur is through the auto-oxidation of amphotericin B bound to membrane components (37).

It is well established that oxidative cell injury is associated with multiple alterations in cell structure and function. It is also known that different levels of oxidative stress can have entirely different effects on cells, ranging from no apparent effect to the stimulation of cell proliferation, growth arrest, and apoptosis and necrotic cell death. Interestingly amphotericin B has been observed to increase *C. albicans* colony-forming units (plating efficiency) when exposed to 0.01 μg/mL (10). The authors were unable to offer an explanation for this observation. Our work with amphotericin B has also shown that the agent is capable of both growth arrest and cell death (48).

The obvious explanation for the elevated XTT reduction, observed when *C. albicans* was exposed to between 0.4 and 1 μg/mL of amphotericin B, was that the increased metabolic activity was required as part of the recovery and resuscitation process that occurred later. However, there is another explanation that may contribute to the oxidative damage hypothesis. The expression and activity of the fungal electron transport chain and respiration are known to be affected by oxidative stress (29). Human cells have been shown to increase tetrazolium reduction in response to oxidative stress and cell cycle arrest (38, 70). It is possible that a similar mechanism occurs in the amphotericin B-exposed *C. albicans*. Yeast and other simple eukaryotes contain components of a proapoptotic pathway which are silent under normal conditions but can be activated by oxidative stress or by manifestation of mammalian death genes, such as *BAK* or *BAX* (20, 41). Exposure of *S. cerevisiae* to low doses of hydrogen peroxide or the accumulation of reactive oxygen species induces programmed cell death in yeast (20). It is interesting to speculate whether amphotericin B could actually have a much more complicated method of action than is currently understood given that the agent has been used to induce programmed cell death in the unicellular protozoan parasite *Leishmania* (39). Amphotericin B has also been recently shown to

induce apoptosis in the human kidney in a dose-dependent manner (82).

VII. FUTURE DIRECTIONS

Compared to bacterial infections, fungal pathogens are more difficult to diagnose, less amenable to treatment, and are associated with the highest attributable mortality (57, 61, 62, 67). *Candida* is presently one of the most common bloodstream pathogens in the United States and Canada (61). Considering the opportunistic nature of *C. albicans* and the age structure of the North American population, there is every reason to believe that this species will present an even greater problem in the near future.

There are several lines of research that can be developed from the work we have done to date. Additional work looking at other methods to evaluate viability could be pursued if there were an interest in further characterizing the interaction between amphotericin B and *C. albicans* or even between different species of microorganisms and antimicrobial agents. Analyzing the morphological and physiological properties of individual *C. albicans* cells using a flow cytometer would be very useful to better characterize the cellular condition. Many of the assays developed to study viability in yeast were initially optimized with flow cytometry in mind. It would be useful to be able to further characterize a

heterogeneous population of cells exposed to a concentration of antifungal agent by size and also the amount of vitality- and mortality-specific fluorescence intensity. Cells that responded to a given resuscitation condition could be sorted out and plated to see if increased vitality as measured with vitality dyes could predict the ability to replicate on agar.

We have performed an analysis of protein expression of cells exposed to amphotericin B using one-dimensional polyacrylamide gel electrophoresis and were unable to resolve differences between whole cell lysates. This line of research could be pursued further using two-dimensional polyacrylamide gel electrophoresis to assess protein expresssion or evaluate mRNA expression with Northern blotts.

Additional work could be done using antibodies directed against specific heat shock proteins in combination with polyacrylamide gel electrophoresis. It would also be very interesting to evaluate the physiology of *C. albicans* cells at the level of gene expression at different points using DNA microarray technology. There are number of commercial labs that offer this service for the *C. albicans* genome. By evaluating the genome-wide response that occurs with resuscitation at 22°C, it might be possible to make a connection to how this process could take place *in vivo* at 37°C. The resuscitation process could then

be replicated using "*in vivo* triggers" one would expect to find in the human host during a systemic infection.

Additional research can be performed to better understand the resuscitation mechanism demonstrated in *C. albicans* and to chose antifungal agents that can be used in humans to prevent it. This research lends itself well to the use of *in vivo* studies with the rabbit animal model (83) to evaluate the improvements and synergy seen *in vitro*. The conversion from sublethal injury to lethal injury after sequential treatment with amphotericin B followed by miconazole also suggests that work looking into the sequential use of amphotericin B with other agents should be investigated. Our work has suggested that sequential use of amphotericin B followed by another antifungal agent can improve the killing with amphotericin B and this needs to pursued further.

When evaluating antifungal inhibition, a more in-depth evaluation of the physiological mechanisms involved in fungal vitality and mortality should prove useful. The potential for using our methods to evaluate new antifungal drugs and combinations represents a powerful tool already being used with some success by others. One obvious target that stands out upon consideration of our work is the fungal cell mitochondria. Although the concerns of toxicity to the human cell would

be great it is possible that even an agent that causes a low level of toxicity in humans could have a huge impact on treatment of infection. Sequential treatment with a mitochondrial inhibitor could remove the maintenance energy in sublethally injured *C. albicans* cells and produce physiological conflict within these cells resulting in the loss of an essential rescue mechanism.

The insights learned from the our work can also be used in combination with the assays that were developed to improve antifungal susceptibility testing for the clinical microbiology laboratory with the aim of having a direct impact on the improvement of patient treatment.

VIII. CONCLUSIONS

1. Yeast cell death after exposure to amphotericin B is a gradual process that can be delineated into different stages between alive and dead. The loss of replication and the ability to form CFUs is an early event in this process.

2. *C. albicans* is able to exist in a viable nonreplicating state that is capable of resuscitation and growth after additional incubation for 15 hr at 22°C.

3. The capacity of *C. albicans* to recover and grow after treatment with amphotericin B at serum concentrations observed in patients with conventional intravenous doses indicates that the agent may not be fungicidal as it is currently used.

4. Sequential combination therapy with amphotericin followed with a second antifungal agent may prove to be effective at achieving true fungicidal inhibition of *C. albicans* and should be pursued with a more thorough survey of drugs that could be used subsequent to amphotericin B.

5. When evaluating antifungal inhibition, a more in-depth evaluation of the physiological mechanisms involved in fungal vitality and mortality should be considered using assays that directly and separately assess these parameters.

6. The measurement of *C. albicans* intracellular ATP has considerable promise as a rapid method for obtaining susceptibility results for amphotericin B, with results as early as 30 minutes.

7. The CFDA-modified microdilution method provides objective and quantifiable endpoints for antifungal susceptibility testing at 24 and 48 h that are reproducible and easy to interpret.

8. The low-high phenotype strains of *C. albicans*, that exhibit exacerbated trailing-growth in the presence of fluconazole and produce discordant MICs that are susceptible at 24 h and resistant at 48 h, are susceptible using a CFDA-modified microdilution method, an ATP macrodilution method and absorbance at 24 and 48 h. The low-high phenotype is an artifact of the conditions used for susceptibility testing.

IX. FIGURES

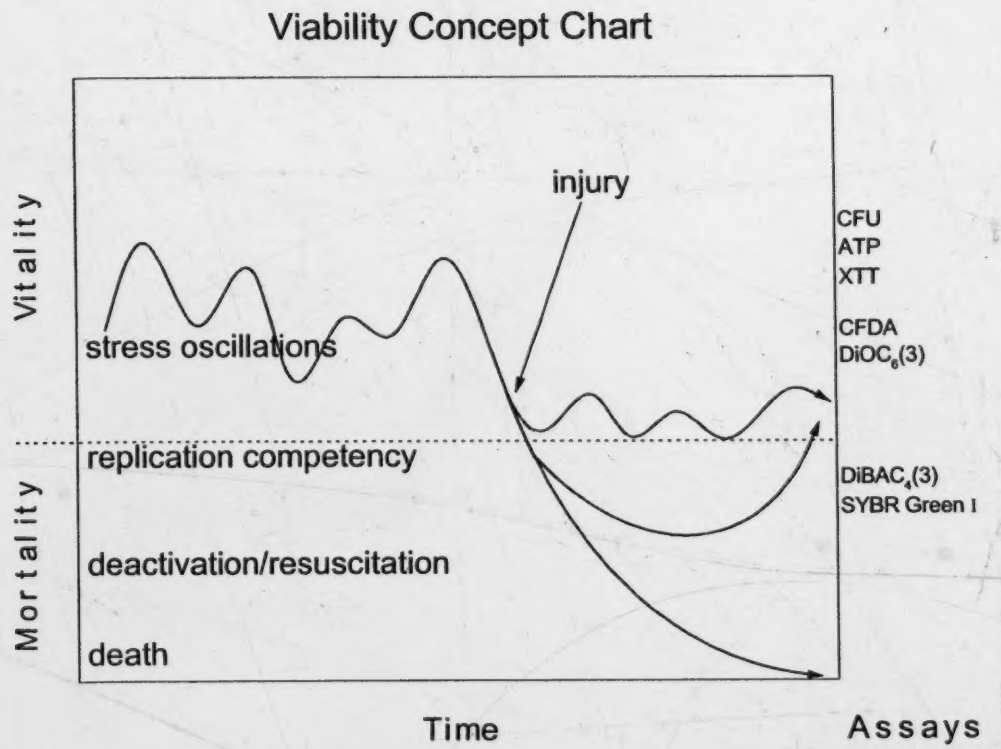

Fig. 7.1. A concept chart for *C. albicans* viability. A yeast cell has the capacity to survive minor stresses and oscillate over time between an energized and deenergized state. Larger perturbations can result in the loss of replication competency of the cell, followed by stepwise changes in its physical-chemical state. The result can be deactivation leading to cell death or resuscitation and resumption of growth. Direct estimations of vitality and mortality, utilizing different assays to evaluate different parameters, permits the development of an overview of 'true viability'.

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Appendix I

MATERIALS AND METHODS

I. Antifungal drugs.

Analytical-grade powders of the six antifungal drugs used in the work described here were provided by the manufacturers: amphotericin B (Bristol Myers-Squibb Co., Princeton, N. J.); fluconazole and voriconazole (Pfizer, Inc., Groton, Conn.); ketoconazole and itraconazole (Research Diagnostics, Inc., Flanders, N. J.); and flucytosine (Hoffmann-La Roche, Inc., Nutley, N. J.). Stock solutions of fluconazole and flucytosine were prepared in distilled water, and those of amphotericin B, itraconazole, voriconazole, and ketoconazole were prepared in 100% dimethyl sulfoxide (3). Stock solutions of antifungal agents were made to 10,000 μg/ml and frozen at –70°C. Each of the stock solutions was thawed once and fresh dilutions were used.

II. Fungal culture.

Yeasts are able to grow on routine bacteriological media, such as blood agar plates. However, if yeasts are present together with a bacterial population, it is possible that the bacteria will suppress yeast

growth. It is therefore understood that a selective medium such as Sabouraud agar should be used for cultivating clinical yeast specimens (3). Sabouraud dextrose agar and variations of it differ from conventional bacteriological media in having a high carbohydrate content (3% dextrose or sucrose) and an acidic pH (approximately 4.0). Both of these conditions are inhibitory to most bacteria (2).

In accordance with the standardized protocol for inoculum preparation in the NCCLS M27-A reference method, all *C. albicans* strains were subcultured from sterile vials of double strength skim milk onto Sabouraud dextrose agar (Difco, Sparks, Md.) (3). Strains were passaged twice at 35°C to ensure purity and viability before being used. The culture samples were grown on SDA plates at 35°C for 48 h for plate counting. Samples were plated in triplicate after appropriate serial dilutions in 0.85% physiological saline. When performing resuscitation experiments, agar plates could be kept up to a month in plastic bags at 35°C.

Liquid media used for growing of *C. albicans* included RPMI 1640 broth medium and YPD broth medium. RPMI 1640 is completely synthetic medium composed of 39 constituent nutrients that is adjusted to pH 7.0 with MOPS (3-[N-morpholino] propanesulfonic acid) buffer (Angus buffers & Biochemicals, Niagara Falls, N. Y.). RPMI 1640 is used to

help reduce variability with antifungal susceptibility testing, although the composition of the media does vary depending on the commercial source. YPD is a neutral nonsynthetic broth media composed of 1% yeast extract, 1% mycological peptone and 3% D-Glucose.

III. REFERENCE LIST

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